Activation of phospholipase D by G protein-coupled receptors

by

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This Thesis is dedicated to my mother Dorothy, sister Eileen and grandfather Peter who I think would have thought of me as a good each way bet on reaching the finishing line.

I declare that the studies presented in this thesis are the result of my own independent investigation with the exceptions of the production of clones of CHO cells stably expressing VIP and PACAP receptors and the construction of chimaeric VIP_2 and PACAP receptors which was done by Dr Eve Lutz. cAMP measurements on the VIP_1, VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors expressed in CHO cells were done by Mel Johnson and the iodination of receptor ligands was done by John Bennie and Sheena Carroll. The measurement of PLD activity in anterior pituitary tissue, the immunoprecipitation of proteins from solubilized membranes from cell lines with antisera to the M_3 and AT_1 receptors, and ARF1/3 and RhoA proteins and [^{3}H]N-methyl scopolamine and [^{125}I]buserelin binding was carried out by Rory Mitchell.

This work has not and is not currently being submitted for any other degree or professional qualification.

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ABSTRACT

The activation of phospholipase D (PLD) by the G protein-coupled receptor (GPCR) family was investigated with special attention paid to the interaction of receptors with small G proteins such as ADP-ribosylation factor (ARF) and RhoA. Agonist-stimulated activation of PLD by the M_3 muscarinic, B_2 bradykinin and H_1 histamine receptors was sensitive to brefeldin A (BFA), an inhibitor of guanine-nucleotide exchange on ARF. In contrast the thrombin and thromboxane A_2 receptors stimulated a PLD response insensitive to BFA. The Rho inhibitor C3 exoenzyme from *Clostridium botulinum* and a negative functional construct of RhoA markedly reduced PLD activity stimulated by the M_3 muscarinic but not the thrombin receptor. The receptors investigated which couple to PLD by a mechanism involving ARF all contain in their seventh transmembrane domain (TMD 7), the amino acid sequence $AsnProX_{2-3}Tyr$ (where X is any amino acid). In contrast, the receptors such as thrombin which activate PLD in an ARF-independent manner have an $AspProX_{2-3}Tyr$ motif. The importance of this highly conserved motif was further indicated by studies on the mouse gonadotropin-releasing hormone (GnRH) receptor which contains the $AspProX_{2-3}Tyr$ sequence and a mutant form of the GnRH receptor where the aspartate at position 318 is replaced with an asparagine thereby restoring the $AsnProX_{2-3}Tyr$ motif present in the majority of group I GPCRs. The wild-type GnRH receptor stimulated PLD in a BFA-insensitive manner, while the Asn318 mutant GnRH form gained BFA-sensitivity. The co-immunoprecipitation of the agonist-treated Asn318 mutant but not wild-type GnRH receptor using antibodies raised to ARF 1/3 and RhoA indicated that a direct physical association between the receptor and small

G proteins occurs and appears to be dependent on the presence of the $AsnProX_{2-3}Tyr$ motif. In a similar fashion the M_3 receptor could be immunoprecipitated using antibodies to ARF 1/3 and RhoA as shown by specific [^{3}H]N-methyl scopolamine binding. Immunoreactive ARF and Rho could correspondingly be immunoprecipitated using antibodies to the M_3 receptor or the similarly $AsnProX_{2-3}Tyr$ containing AT_1 angiotensin receptor. Receptors from group II, the VIP/PACAP/calcitonin family of GPCRs were also shown for the first time to activate PLD in a potent agonist-induced manner. The VIP_1, VIP_2 and $PACAP_{long}$ receptors all activated PLD in a BFA-sensitive fashion. However the $PACAP_{short}$ receptor coupled to PLD in an ARF-independent manner which involved phospholipase C (PLC), as shown by its sensitivity to the PLC inhibitor U73122. The $PACAP_{long}$ receptor possesses an extra 28 amino acid "hop-1" cassette in the third intracellular loop (i3). The importance of the hop-1 cassette and the surrounding i3 in receptor coupling to PLD was studied using chimaeric receptor constructs comprising the VIP_2 receptor with the i3 loop replaced by that of either the $PACAP_{short}$ or $_{long}$ receptor. The $VIP_2/PACAP_{long}$ receptor chimaeric displayed a BFA-sensitive activation of PLD not seen with the $VIP_2/PACAP_{short}$ receptor construct.

Thus activation of PLD by a number of receptors of the GPCR family involves their association with small G proteins of the ARF and Rho families and distinct receptor domains such as TMD 7 and i3 have been shown to act as crucial determinants of receptor/small G protein coupling.

ABBREVIATIONS

^3H	tritium
^{125}I	iodine 125 radioisotope
5-HT	5-hydroxytryptamine
ARF	ADP-ribosylation factor
B_2	B_2 bradykinin receptor
β_2	β_2-adrenergic receptor
BeF_3^-	beryllium fluoride
BFA	brefeldin A
cAMP	adenosine 3′,5′-cyclic monophosphate
BSA	bovine serum albumin
C3 exoenzyme	*Clostridium botulinum* ADP-ribosyltransferase
CO_2	carbon dioxide
Ca^{2+}	calcium ion
CHO	Chinese hamster ovary
Ci	curies
CTx	cholera toxin
cDNA	complimentary deoxyribonucleic acid
Da	daltons
DAG	diacylglycerol
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified Eagle medium
DMF	dimethylformamide
DMSO	dimethylsulphoxide
dpm	disintegrations per minute
EDTA	sodium ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(b-aminoether) N, N, N',N'-tetra-acetic acid
GTPγS	guanosine 5′-O-(3 thio)-triphosphate
$GPPCH_2P$	guanosine 5′-[βγ-methylene] triphosphate
G protein	gaunine-nucleotide binding protein
GAP	GTP'ase activating protein
GDP	guanine diphosphate
GTP	guanine trisphosphate
GnRH	gonadotropin hormone-releasing hormone
H_1	H_1 histamine receptor

InsP	inositol phosphates
LH	luteinising hormone
lysoPA	lysophosphatidic acid
M	Molar
M_3	M_3 muscarinic receptor
MAP kinase	mitogen-activated protein kinase
MEM	minimum essential medium
min	minute(s)
ml	millilitres
mM	millimolar
PA	phosphatidic acid
PACAP	pituitary adenylate cyclase-activating polypeptide
PAF	platelet-activating factor
PtdCho	phosphatidylcholine
PDBu	phorbol 12,13-dibutyrate
PIP_2	phosphatidylinositol 3,5-biphosphate
PIP 5K	phosphatidylinositol 4-phosphate 5-kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PPH	phosphatidic acid phosphohydrolase
PTx	pertussis toxin
TMD	transmembrane domain
Tris	tris (hydroxymethyl) aminoethane
TXA_2	thromboxane A_2 receptor
µM	micromolar
v/v	volume by volume
VIP	vasoactive intestinal peptide

Bmax	maximum number of ligand binding sites/mg of protein
EC_{50}	concentration required to evoke 50% of the max response
IC_{50}	concentration required to inhibit 50% of the response

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Some of the results presented in this Thesis have been published as follows:

R. Mitchell, D. McCulloch, E. Lutz, M. Johnson, C. MacKenzie, M. Fennell, G. Fink, W. Zhou, and S. C. Sealfon (1998) Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. *Nature* **392** pp 411-414

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CHAPTER 1

Introduction

A large number of hormones, neurotransmitters, chemokines, local mediators, and sensory stimuli exert their effects on cells and the organism by binding to guanine nucleotide-binding (G) protein-coupled receptors (GPCRs). Almost 2000 such receptors are known, and more are being discovered all the time. Despite the wide range of ligands that activate these receptors, the receptors themselves share a surprising amount of structural homology, both primary and secondary. The overall structural features of the GPCR family are highly conserved: all of these receptors contain seven hydrophobic domains, postulated to span the plasma membrane, connected by hydrophilic extracellular and intracellular loops.

The tertiary organisation of these structures is also thought to be highly conserved. The breakdown of phospholipids is now globally recognised as an important mechanism used by GPCRs to transduce the signal of receptor stimulation. In this context the breakdown of phosphatidylcholine by phospholipase D has emerged as a major pathway by which class I (rhodopsin) family GPCRs may regulate the activity of the cell. The activation of PLD has been associated with the respiratory burst in neutrophils, control of protein trafficking and secretion, mitogenesis and changes in cell morphological and motility (Boccking, S. B. and J. H. Exton 1996, Exton, J. H. 1997). However the elucidation of the mechanism of PLD activation has moved slowly; hampered by the difficulty in purifying the enzyme to homogeneity. Recently small G proteins of the ADP-ribosylation factor (ARF) and Rho families have been shown to function as activators of partially purified PLD (Bowman, E. P., et al. 1993, Brown, H. A., et al. 1993, Cockcroft, S., et al. 1994), and the cloned PLD1 enzyme (Hammond, S. M., et al. 1995, Hammond, S. M., et al. 1997, Park,

S.-K., et al. 1997). Despite recent advances in resolving the regulators and co-factors that are involved in the activation of PLD, the mechanism by which GPCRs utilise these activators is still poorly understood. Furthermore the question of stimulation of PLD by other families of GPCRs has been almost completely overlooked.

1.1 AIMS OF THIS STUDY

The primary aim of this study was to determine the method of phospholipase D (PLD) by G-protein coupled receptors (GPCRs) from class I and II. Receptors from class I which are characterised by their coupling to the G protein G_q, and ability to activate phospholipase C (PLC) and thus the breakdown of phosphatidylinositol 4,5-bisphosphate were studied. The activation of PLD by receptors for Vasoactive Intestinal Peptide (VIP) and Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP), which belong to class II and couple tightly to G_s, was also examined. The main questions addressed in this project have been:

- What is the mechanism of PLD activation by selected class I GPCRs: is there an involvement of the small G proteins ARF and RhoA and PLC?

- Does the highly conserved Asn/Asp-Pro-X_{2-3}Tyr motif in class I receptors have any influence on receptor coupling to PLD?

- Is there a close association between class I GPCRs and ARF and RhoA?

- Do the class II receptors; VIP_1, VIP_2 receptors and long and short splice variants of the PACAP receptor stimulate PLD and what is the mechanism of activation?

- What is the role if any of the third intracellular loop of the $PACAP_{short}$ and $_{long}$ receptors in coupling to PLD?

1.2 G PROTEIN-COUPLED RECEPTORS

Almost 2000 G protein-coupled receptors have been reported since the cloning of bovine rhodopsin in 1983 (Nathan, J. and D. S. Hogness 1983) and the β-adrenergic receptor in 1986 (Dixon, R. A., et al. 1986). All GPCRs have an extracellular N-terminal segment, seven α-helical transmembrane domains (TMDs), three extracellular and intracellular loops, and an intracellular C-terminal tail, which can form a fourth cytoplasmic loop when the C-terminal segment is palmitoylated on cysteine and the modification allows membrane association. GPCRs have been classified by their sequence homology, ligand structure and receptor function into 3 major subfamilies; the rhodopsin (class I), VIP/PACAP/secretin (class II) and the metabotropic glutamate receptor (mGluR) family, which includes the Ca^{2+}-sensing receptor (class III) (Conn, P. J. and J.-P. Pin 1997). The class I family have been the focus in the investigation of receptor function, with the rhodopsin, adrenergic, and muscarinic acetylcholine receptors being the most extensively studied. The VIP/PACAP/secretin and the metabotropic glutamate family have been relatively neglected in comparison to the class I family, although the basic signal transduction characteristics of class II and III receptors has been studied. The class I and II families have been the subject of this study, with the mechanism of receptor coupling to PLD partially characterised. Receptors are proposed to consist of two functional regions, the ligand binding domain involving the N-terminal segment, extracellular loops and/or the pocket formed by the 7 TMDs. In addition there is the G protein-binding domain, which is generally considered to be defined by the intracellular loops (and perhaps elements of the transmembrane domain close to the intracellular surface).

1.2.1 Heterotrimeric G proteins

G proteins are composed of α, β and γ subunits and although there are numerous gene products encoding each subunit (20 α, 6 β, and 12 γ gene products are known). There are four main classes of G proteins defined by the interaction of the α subunits with their effectors. These are: G_s, which activates adenylate cyclase; G_i, which inhibits adenylate cyclase; G_q, which activates PLC; and G_{12} and G_{13}, which are of unknown function (Hamm, H. E. 1998). G_{12} and G_{13} have been implicated in the control of DNA synthesis (Aragay, A. M., et al. 1995), activation of Jun kinases (Prasad, M. V., et al. 1995) and the control of the actin cytoskeleton in a Rho-dependent manner (Bühl, A. M., et al. 1995). G_{12} and G_{13} have been shown to modulate the action of a Rho-specific exchange factor. (Hart, M. J., et al. 1998, Kozasa, T., et al. 1998). Within the four major classes of G proteins there are closely related proteins with divergent functions. A G protein G_{olf}, is a member of the G_s family and is stimulated by odorant receptors and activates adenylate cyclase. The G_i family also contains G_o, G_t, G_z, G_{12} and G_{gust} proteins. G_o proteins regulate calcium and potassium channels, G_t (transducin), is the G protein that is stimulated by the rhodopsin receptor and activates cGMP phosphodiesterase and G_z proteins inhibit adenylate cyclase. Within the G_q family (G_q, G_{11}, G_{14}, G_{15} and G_{16}) all members activate PLC (Conklin, B. R. and H. R. Bourne 1993, Hamm, H. E. and A. Gilchrist 1996).

G proteins are inactive in the GDP-bound, heterotrimeric state and are activated by receptor-catalysed guanine nucleotide exchange resulting in GTP binding to the α subunit. GTP binding leads to the dissociation of the Gα-GTP from Gβγ subunits and the stimulation of downstream effectors by both activated Gα-GTP and free Gβγ subunits (Clapham, D. E.

and E. J. Neer 1997, Hamm, H. E. 1998). In the past, the βγ subunits of heterotrimeric G proteins were not recognised as distinctly important in the signal transduction processes stimulated by agonist. Recently however the influence of βγ subunits on numerous targets has been extensively described. Once dissociated from Gα, βγ subunits stimulate PLCβ1-3 (Camps, M., et al. 1992, Sternweis, P. C. 1994), PLA_2 (Jelsema, C. L. and J. Axelrod 1987) inward rectifying potassium channels (Logothetis, D. E., et al. 1987), phosphatidylinositol 3-kinase (PI3-K) (Stephens, L., et al. 1994), G protein-coupled receptor kinases (GRKs) (Pitcher, J. A., et al. 1992) and the MAP kinase cascade (Crespo, P., et al. 1994, Hawes, B. E., et al. 1995, Koch, W. J., et al. 1994, van Biesen, T., et al. 1995).

The coupling of GPCRs to the mitogenic Ras/MAPK signalling pathway through βγ subunits has been observed for many of the G_i-coupled receptors such as the thrombin, lysoPA and α_2-adrenergic receptors (Alblas, J., et al. 1993, Hordijk, P. L., et al. 1994, Howe, L. R. and C. J. Marshall 1993, Moolenaar, W. H. 1995, van Corven, E. J., et al. 1993). However agonists also acting on G_s-, G_q-, along with G_i-coupled receptors stimulated MAPK, and both the Gα and Gβγ subunits were implicated in the signalling processes (Della Rocca, G. J., et al. 1997, Faure, M., et al. 1994, Wan, Y. and X.-Y. Huang 1998). Activation of the MAP kinase cascade by receptors (including GPCRs) often starts with the tyrosine phosphorylation of an adaptor protein, SH2-domain-containing α2-collagen related (Shc), which then associates with growth-factor-receptor-bound-protein 2 (Grb2) and son of sevenless (SOS) proteins through their SH2 and SH3 domains. Sos is a cytoplasmic guanine-exchange factor and stimulatory of Ras function. The phosphorylation of the Shc adaptor protein and the formation of the Shc-Grb2-Sos1 complex has been

reported in the activation of MAPK by G_i and G_q-linked receptors. In addition, there is evidence for a separate PKC-dependent/$p21^{ras}$ -independent pathway for the activation of MAPK by G_q-linked receptors (reviewed in (Denhardt, D. T. 1996, van Biesen, T., et al. 1995, van Biesen, T., et al. 1996)).

1.2.2 Rhodopsin family (class I) GPCRs

Although no high-resolution structure of a GPCR has yet been determined, a recently obtained low resolution electron diffraction structure of rhodopsin, a model GPCR, shows the position and orientation of the 7 TMDs (Baldwin, J. M. 1993, Baldwin, J. M. 1994, Schertler, G. F. X., et al. 1993, Unger, V. M., et al. 1997). According to the Baldwin model , TMDs 4, 6 and 7 are nearly perpendicular to the plane of the membrane, whereas TMDs 1, 2 and 3 are tilted (Schertler, G. F. X. and P. A. Hargrave 1995, Schertler, G. F. X., et al. 1993, Unger, V. M. and G. F. X. Schertler 1995). The use of site-directed mutations, site-directed spin labelling techniques and nuclear magnetic resonance are beginning to define structures for intracellular loops in several receptors. In rhodopsin and muscarinic receptors, all three approaches indicate that the amino-terminal residues of the third cytoplasmic loop (i3) form α-helical extensions of TMD 5. Some evidence suggests that the carboxy-terminal residues of i3 immediately preceding TMD 6 are also α-helices (Blüml, K., et al. 1994, Burstein, E. S., et al. 1995, Hill-Eubanks, D., et al. 1996, Yang, K., et al. 1996). Both mutagenesis and biochemical experiments using the labelling of rhodopsin and β-adrenergic receptor residues with fluorescent probes suggest that when a receptor is activated by the binding of ligand, there are changes in the relative orientations of the transmembrane helices (Altenbach, C., et al. 1996, Bukusoglu, G. and D. D. Jenness 1996,

Farahbakhsh, Z. T., et al. 1993, Farahbakhsh, Z. T., et al. 1995, Farrens, D. L., et al. 1996, Gether, U., et al. 1997). There is proposed to be a 30° twist in TMD 6 and this change in the orientation of the TMDs is suggested to unmask regions of the receptor that can interact with G proteins. These movements of the TMDs will change the conformation of the i3 loop which connects TMDs 5 and 6, the region of the receptor that has been shown by studies using mutagenesis and chimaeric receptors to determine G protein coupling (Dohlman, H. G., et al. 1991, Gudermann, T., et al. 1996, Savarese, T. M., et al. 1992, Strader, C. D., et al. 1994).

1.2.3 Multiple signals from GPCRs

Many GPCRs can couple to more than one G protein and thus evoke diverse intracellular signals. The thrombin receptor is a good example of this behaviour with evidence for coupling to proteins from the G_q, G_i and G_{12} families (Offermans, S., et al. 1994). M_1 and M_3 muscarinic receptors can similarly couple to G_q and G_i (Offermanns, S., et al. 1994). However the thyrotropin releasing hormone (TRH) receptor can couple to all four G protein families (G_s, G_i, G_q, and G_{12}) (Laugwitz, K. L., et al. 1996).

The existence of related receptor subtypes introduces a further level of versatility into the generation of second messengers by one stimulus; examples are the 12 mammalian 5-hydroxytryptamine (serotonin), 10 adrenergic and 5 muscarinic receptor subtypes identified by molecular cloning (Hoyer, D., et al. 1994, Watson, S. and S. Arkinstall 1994). Furthermore, alternatively spliced variants of receptors can lead to alternative signalling as in the case of the dopamine D2 receptor, which exists in a short and long form. The introduction of 29 amino acids into

the i3 of the D_{2short} receptor which couples to G_{i3}, generates a longer splice variant which displays a preferential coupling to G_{i2} (Guiramand, J., et al. 1995, Monsma, F. J., et al. 1989). Similarly a longer splice variant of the turkey β_1-adrenergic receptor does not readily desensitize or downregulate in response to agonist as is observed with the normal form of the receptor (Hertel, C., et al. 1990).

1.2.4 Desensitization of GPCRs

The control of receptor desensitization is probably the most intensively investigated aspect of GPCR signalling. This is the waning responsiveness of a GPCR in the face of persistent stimulation (Ferguson, S. S. G., et al. 1996, Hausdorff, W. P. 1990). There are three families of regulatory proteins that have been clearly implicated in the process of desensitization of heptahelical receptors: the second messenger-regulated kinases (e.g. protein kinase A (PKA), and PKC), G protein-coupled receptor kinases (GRKs) (e.g. βARK, rhodopsin kinase) and the arrestins (visual and non-visual). The phosphorylation of GPCRs by PKA or PKC on the C-terminal tail or i3 (Hausdorff, W. P. 1990, Sibley, D. R., et al. 1987) and the resulting impairment of receptor coupling to G protein is seen as heterologous desensitization as it is not agonist-specific. In such cases, as any stimulant that elevates cAMP or DAG levels can activate PKA and PKC and thus potentially desensitise any GPCR containing an appropriate PKA and/or PKC consensus phosphorylation site.

Homologous desensitization of GPCR-mediated responses involves the phosphorylation of the agonist-occupied receptor by a GRK and then the binding of an arrestin protein which blocks receptor-G protein coupling (Ferguson, S. S. G., et al. 1996, Inglese, J., et al. 1993, Lefkowitz, R. J. 1998).

There are 6 members of the GRK family at the moment and at least 6 members of the arrestin family. The recruitment of GRKs to the receptor (GRKs 2 and 3 are cytosolic proteins) is mediated by the βγ subunits of the trimeric G protein activated upon agonist stimulation (Daaka, Y., et al. 1997, Lefkowitz, R. J. 1998). The effects of the different GRKs appear to be receptor–specific as shown by the selective desensitization of the β-adrenergic but not the angiotensin II receptor in cardiac cells overexpressing GRK5 (Rockman, H. A., et al. 1996). Furthermore a GRK2/Gβγ complex forms after lysoPA and β-adrenergic, but not thrombin receptor stimulation (Daaka, Y., et al. 1997). Also there appears to be a selective interaction between different Gβγ complexes and GRK isoforms; the carboxy terminus of GRK2 binds Gβ1 and Gβ2, but not Gβ3, while GRK3 binds all three (Daaka, Y., et al. 1997).

1.2.5 VIP/PACAP/secretin family (class II) GPCRs

This new family of receptors (Segre, G. V. and S. R. Goldring 1993) binds small peptide ligands and share characteristics which also make them distinct from the class I family of GPCRs, with which they share less than 12% homology. However work by Donnelly shows that the arrangement of the transmembrane domains in the class II family is likely to be almost identical to the arrangement of rhodopsin family TMDs as reported by Baldwin (Baldwin, J. M. 1993, Baldwin, J. M. 1994, Donnelly, D. 1997). In the course of this project the activation of PLD by the VIP_1 (Ishihara, T., et al. 1992), VIP_2 (Lutz, E. M., et al. 1993), $PACAP_{short}$ and $PACAP_{long}$ receptors (Hashimoto, H., et al. 1993, Hosoya, M., et al. 1993, Morrow, J. A., et al. 1993, Pisegna, J. R. and S. A. Wank 1993, Spengler, D., et al. 1993, Svoboda, M., et al. 1993), was studied. The short and long suffixes for the PACAP receptor refer to two of the 6 splice variants of the PACAP

receptor which were cloned by Spengler and colleagues (Journot, L., et al. 1995, Spengler, D., et al. 1993). The PACAP receptor is found in a short form and 5 longer variants which contain either singly or in combination to produce 28 amino acid inserts in the i3 of the receptor. There are 3 possible inserts; hop-1, hop-2 and hip which can be found individually within the receptor i3, and two receptors which carry a combination of either hop-1-hip, or hop-2-hip. The $PACAP_{long}$ receptor used here contains the 28 amino acid hop-1 insert.

The other receptors within this family include the receptors for the following: glucagon (Jelinek, I. J., et al. 1993), glucagon-like peptide-1 (Thorens, B. 1992), calcitonin (Lin, H. Y., et al. 1991), calcitonin gene-related peptide (Aiyar, N., et al. 1996), gastric inhibitory peptide (Usdin, T. B., et al. 1993), secretin (Ishihara, T., et al. 1991), parathyroid hormone, parathyroid hormone-related peptide (Jüppner, H., et al. 1991), growth hormone-releasing hormone (Mayo, K. E. 1992), corticotrophin-releasing factor (Chang, C. P., et al. 1993), insect diuretic hormone (Regan, J. D. 1994), and two more distantly related receptors, the EMR1 (EGF module-containing mucin-like hormone receptor) (Baud, V. 1995) and the leukocyte activating antigen CD97 receptor (Hamann, J. 1995).

VIP was isolated from small intestine in 1970 (Said, S. I. and V. Mutt 1970), and PACAP from ovine hypothalamus during the search for a hypophysiotrophic factor (Miyata, A., et al. 1989, Miyata, A., et al. 1990). PACAP exists in two amidated forms, PACAP-38 and a shorter form PACAP-27, which contain 38 and 27 amino acids respectively (Miyata, A., et al. 1989, Miyata, A., et al. 1990). The VIP_1, VIP2, $PACAP_{short}$ and $PACAP_{long}$ receptors, in common with the rest of the class II family of GPCRs, activate adenylate cyclase (Ishihara, T., et al. 1992, Lutz, E. M., et al.

1993, Morrow, J. A., et al. 1993, Spengler, D., et al. 1993, Usdin, T. B., et al. 1994). The VIP and PACAP peptides are closely related to each other (51% homology) (Arimura, A. and S. Shioda 1995, Miyata, A., et al. 1990). PACAP and VIP share binding sites in a variety of tissue types. These polypeptides bind to two major groups of sites: type I sites, which prefer PACAP-38 and PACAP-27 over VIP, and type II sites which have approximately equal high affinity for PACAP-38, PACAP-27 and VIP (Arimura, A. 1992, Christophe, J. 1993, Rawlings, S. R. and M. Hezareh 1996). The VIP_1 and VIP_2 receptors are equivalent to type II sites, whereas PACAP receptors prefer PACAP-38 and -27 over VIP, like the type I sites. The ability of the peptides to interact with more than one receptor subtype may be important *in vivo*, by providing another layer of receptor control.

Despite the obvious differences in receptor primary sequence as compared to class I receptors, the class II family appear to couple to many of the same signal transduction pathways, and these will be discussed in Chapter 5. The regulation of receptor function shares many parallels with the rhodopsin family including the importance of receptor phosphorylation in desensitization of the second messenger response e.g. heterologous desensitization of the glucagon-like peptide-1, secretin and glucagon receptors (Buggy, J. J., et al. 1997, Holtmann, M. H., et al. 1996, Widmann, C., et al. 1996). There was also a recent report of the phosphorylation of the secretin receptor by GRKs 2 and 5, and the involvement of an arrestin in the desensitization of the receptor-evoked cAMP response (Shetzline, M. A., et al. 1998). The two families of receptors may therefore be functionally comparable.

1.3 PHOSPHATIDYLCHOLINE BREAKDOWN

Phosphatidylcholine (PtdCho) is the principal phospholipid class in mammalian tissues and constitutes up to 50% of the total cellular phospholipid content (Billah, M. M. and J. C. Anthes 1990, Tronchère, H., et al. 1994). In comparison phosphatidylinositol (PtdIns) represents less than 10% of the total and the preferred substrate of PI-phospholipase C (PLC), phosphatidylinositol 4,5-bisphosphate (PIP_2), accounts for less than 0.5% of phosphatidylinositols (Billah, M. M. and J. C. Anthes 1990, Tronchère, H., et al. 1994). PtdCho-specific phospholipases C (PLC), A_2 (PLA_2) and PLD brings about the release of various lipidic second messengers: unsaturated fatty acids, diacylglycerol or PA, with an associated release of either phosphocholine, lysoPC and choline. These are also involved in the synthesis of PtdCho, therefore in arrangement analogous to that for PtdIns, there is a cycle of PtdCho turnover in cells (Billah, M. M. and J. C. Anthes 1990, Tronchère, H., et al. 1994).

1.4 PHOSPHOLIPASE D

1.4.1 Enzymology of PLD

In addition to the above PtdCho PLD, enzyme activities effective on phosphatidylethanolamine and phosphatidylinositol have also been described. The hydrolysis of phosphatidylethanolamine by a PLD activity has been reported in microsomes prepared from canine brain (Natarajan, V., et al. 1984) and in cultured cells (Kiss, Z. and W. B. Anderson 1989, Kiss, Z. and W. B. Anderson 1989) Balsinde *et al.* have described a PLD activity in human neutrophils and plasma that hydrolysed PtdIns but not

PtdCho (Balsinde, J., et al. 1988, Balsinde, J. and F. Mollinedo 1990). Furthermore a glycosyl-phosphatidylinositol-specific PLD is an enzyme that hydrolyses the inositol-phosphate linkage in proteins such as alkaline phosphatase and acetylcholinesterase, that are anchored to the membrane by a phosphatidylinositol-glycan moiety (Low, M. G. 1989, Low, M. G. and D. B. Zilversmit 1980, Malik, A.-S. and M. G. Low 1986). However the regulation and physiological implications of these activities have not received the attention paid to the PtdCho-specific PLD activity which is the subject of investigation presented here.

Phospholipase D was first described in plants in 1947 (Hanahan, D. J. and I. L. Chaikoff 1947, Hanahan, D. J. and I. L. Chaikoff 1948) and is widespread in the plant kingdom having been purified to homogeneity from cabbage leaves (Abousalham, A., et al. 1993) and castor bean endosperm (Wang, X., et al. 1993). PLD has also been found in simple eukaryotes such as the slime mould *Dictostelium discoideum* (Cubitt, A. B., et al. 1993)and the budding yeast *Saccharomyces cerevisiae* (Ella, K. M., et al. 1995, Waksman, M., et al. 1996). Additionally the prokaryotes *Cornyebacterium pseudotuberculosis* and *Arcanobacterium haemolyticum* express sphingomyelin-specific PLDs (Ceuvas, W. A. and J. G. Songer 1993, Hodgson, A. L. M., et al. 1990). PLD was long thought to be absent from mammalian tissues until its presence was reported in detergent-solubilized rat membranes (Saito, M. and J. Kanfer 1973). PLD catalyses the hydrolysis of phospholipids at their terminal phosphodiester bond, thus producing phosphatidic acid (PA) and the free head group. The enzyme was subsequently partially purified by Kanfer's group and the ability of PLD to catalyse a transphosphatidylation reaction (the transfer of the phosphatidyl moiety from one alcohol to another) was demonstrated

(Kanfer, J. N. 1980, Kobayashi, M. and J. N. Kanfer 1987). In the presence of glycerol or a primary alcohol such as ethanol, propanol or butanol the respective phosphatidylalcohol accumulated at the expense of PA (Kanfer, J. N. 1980). The transphosphatidylation reaction is regarded as unequivocal evidence of PLD activity. Phosphatidic acid produced by the hydrolysis of PtdCho by PLD is further metabolised by phosphatidate phosphohydrolase (PPH) to produce diacylglycerol, an activator of protein kinase C (PKC) (Brindley, D. N. and D. W. Waggoner 1996, Siddiqi, R. A. and J. H. Exton 1992, Smith, S. W., et al. 1957). Although there is some controversy over whether the diglycerides species produced by PLD are good activators of PKC (Hodgkin, M. N., et al. 1998, Pettitt, T. R., et al. 1997). PA can also be hydrolysed by a PA-specific PLA_2 activity thereby producing free fatty acid and lysophosphatidic acid (lysoPA) (Dennis, E. A. 1994), which is suggested to have numerous physiological effects to be discussed later.

The existence of multiple isoforms of PLD has been suggested by numerous biochemical studies investigating the subcellular distribution of PLD enzymes and mechanisms of activation. Massenburg *et al.* purified two PLD activities from rat brain (Massenburg, D., et al. 1994), one was activated by the small G protein ARF and PIP_2 and is most likely related to the ARF and Rho-dependent PLD activity described by other groups (Bowman, E. P., et al. 1993, Brown, H. A., et al. 1995, Brown, H. A., et al. 1993, Cockcroft, S., et al. 1994, Singer, W. D., et al. 1995). This ARF and PIP_2-activated PLD had an Mr of 95,000 as determined by hydrodynamic analysis (Brown, H. A., et al. 1995). The second PLD activity resolved by Massenburg *et al.* was an oleate-activated form (Massenburg, D., et al. 1994) previously identified by others (Chalifa, V., et

al. 1990, Kobayashi, M. and J. N. Kanfer 1987, Siddiqi, R. A. and J. H. Exton 1992), and recently purified (Okamura, S. and S. Yamashita 1994). The oleate-activated PLD appears to be more tightly associated with the membrane than the ARF-activated PLD. The oleate-activated PLD enzyme could not be solubilized from the membrane by high salt concentrations (1 M NaCl) (Danin, M., et al. 1993), whilst the ARF-dependent PLD was solubilized by 0.4 M NaCl (Brown, H. A., et al. 1993). The purified form of this oleate-activated PLD had a M_r of 190,000 (Okamura, S. and S. Yamashita 1994). In addition to the membrane associated PLD isoforms, there have been reports of cytosolic PLD activity in bovine heart and lung, HL-60 cells and human placenta (Bocckino, S. B. and J. H. Exton 1996, Siddiqi, A. R., et al. 1995, Vinggaard, A. M., et al. 1997, Wang, P., et al. 1991).

1.4.2 Cloning and characterisation of PLD enzymes

The cloning of a cDNA for a higher eukaryote PLD was initially reported by Wang *et al.* (Wang, X. M., et al. 1994), who isolated the cDNA for castor bean (*Ricinus communis* L) PLD by using oligonucleotide probes based on the amino-terminal amino acid sequences of the purified protein (Wang, X., et al. 1993). PLD enzymes have subsequently been cloned and sequenced from *Streptomyces antibioticus* (Iwasaki, Y., et al. 1994), rice and maize plants (Ueki, J., et al. 1995). Furthermore the SPO14 gene of *Saccharomyces cerevisiae* reported to be essential for meiosis, was identified as the yeast PLD gene (Ella, K. M., et al. 1996, Honigberg, S. M., et al. 1992, Rose, K., et al. 1995, Waksman, M., et al. 1996).

The first cloned mammalian homologue of a plant PLD was PLD1, and a shorter hPLD1b splice variant has been identified, which lacks the 38

amino acids from 585-624 present in hPLD1a (Hammond, S. M., et al. 1995, Hammond, S. M., et al. 1997). A rat PLD1 (rPLD1) has also been cloned recently and this exhibited similar properties to hPLD1 (Park, S.-K., et al. 1997). This enzyme is membrane associated and activated by PIP_2, ARF, RhoA (and also Rac-1 and Cdc42), and PKC-α, and probably represents the PLD activity reported by Brown and colleagues in rat brain (Brown, H. A., et al. 1993). A second isoform of PLD, PLD2 was recently cloned from mouse (mPLD2) (Colley, W. C., et al. 1997) and rat (rPLD2) (Kodaki, T. and S. Yamashita 1997). The human homolog of PLD2 (hPLD2) has also recently been cloned from a B cell library and partially characterised (Lopez, I., et al. 1998).

The cloning and sequence analysis of human hPLD1 shows that it has none of the recognised Src homology (SH) domains; SH2 and SH3, or the pleckstrin homology (PH) domain (Hammond, S. M., et al. 1995). This is in contrast to the various isoforms of PLC, which contain these domains. Furthermore no similarity exists between hPLD1 and the phosphatidylinositol-glycan-specific PLD or PLC (Hammond, S. M., et al. 1995). However the cloned PLD enzymes contain conserved regions that are homologous to other proteins involved in phospholipid biosynthesis and make them members of a new family of proteins. Comparison of the sequences encoding hPLD1, SPO14 and castor bean PLD showed that they shared four homologous regions denoted I, II, III and IV (Morris, A. J., et al. 1996). The PLD homologue from *Streptomyces* shares some of the conserved sequences present in regions I, III and IV. Moreover portions of the sequences in domains I and IV are found in *Escherichia coli* cardiolipin synthase (CL) and phosphatidylserine synthase (PSS), enzymes that catalyse the final step in phospholipid production. PLD enzymes, CL

and PSS can all catalyse the transphosphatidylation reaction that is characteristic of PLD in mammalian cells (Morris, A. J., et al. 1996). Ponting and Kerr proposed that these proteins are members of members of a wider family that include a pair of vaccinia virus encoded proteins; p37K and K4, an endonuclease (nuc) and a helicase-like protein (o338) (Ponting, C. P. and I. D. Kerr 1996). The p37K protein from vaccinia virus when mutated in the HxKxxxxD motif shared with PLD, is no longer able to efficiently infect cells (Sung, T. C., et al. 1997). The HxKxxxxD motif which is present twice in PLD has been proposed to be involved in the catalytic pathway for the hydrolysis of PtdCho by PLD (Sung, T. C., et al. 1997).

The mouse isoform of PLD2 was reported to be highly active in the presence of PIP_2, both *in vitro* and *in vivo* and could not be further stimulated by ARF or Rho proteins or PKC-α (Colley, W. C., et al. 1997). Similarly the rat isoform of PLD2 was active in the presence of PIP_2, but not ARF or Rho proteins. However the activity was relatively low compared to that reported for mPLD2 (Kodaki, T. and S. Yamashita 1997). Moreover hPLD2 was not highly active and displayed low basal activity, furthermore hPLD2 could be stimulated by recombinant ARF1 in the presence of PIP_2 (Lopez, I., et al. 1998). However, the stimulation of hPLD2 by ARF1 was 8 fold lower than that observed for hPLD1 and there was no stimulation of hPLD2 activity by RhoA, Rac1 and PKC-α (Lopez, I., et al. 1998). The constitutive activity observed for mPLD2 may be due to the loss of inhibitory factors upon purification for use in enzyme assays. When hPLD2 and mPLD2 were expressed in the same Hi5 insect cell line, they exhibited similar basal activities (Lopez, I., et al. 1998). There are uncharacterized inhibitors of PLD2 partially purified from bovine brain

(Colley, W. C., et al. 1997). Other inhibitors of PLD have been described including fodrin, a non-erythroid spectrin protein (Lukowski, S., et al. 1996) and synaptojanin (a phosphatidylinositol 5-phosphatase) (Chung, J.-K., et al. 1997), which appear to reduce levels of PIP_2 (Lukowski, S., et al. 1998, McPherson, P. S., et al. 1996) a co-factor for PLD activation. Also an apparently direct inhibition of PLD was reported for clathrin assembly protein 3 (AP3) (Lee, C., et al. 1997), and arfaptin the ARF-docking protein, inhibits PLD activity perhaps by binding directly to ARF (Tsai, S.-C., et al. 1998). In the study of hPLD1a and 1b, rPLD1 and hPLD2, oleate was inhibitory, suggesting that the oleate-activated form of PLD reported by various groups and purified recently by Okamura *et al.* may still be undiscovered (Chalifa, V., et al. 1990, Kobayashi, M. and J. N. Kanfer 1987, Massenburg, D., et al. 1994, Okamura, S. and S. Yamashita 1994, Siddiqi, R. A. and J. H. Exton 1992).

PLD activity has been found to have a wide distribution within the cell and it has been found on plasma membrane and cytoplasm (Bocckino, S. B. and J. H. Exton 1996, Brown, F. D., et al. 1998, Edwards, J. S. and A. W. Murray 1995, Morgan, C. P., et al. 1997, Provost, J. J., et al. 1996, Whatmore, J., et al. 1996), nucleus (Balboa, M. A. and P. A. Insel 1995), and other membranes such as golgi, mitochondrial and endoplasmic reticulum (ER) (Decker, C., et al. 1996, Edwards, J. S. and A. W. Murray 1995, Ktistakis, N. T., et al. 1995) and secretory vesicles/endosomes (Brown, F. D., et al. 1998, Morgan, C. P., et al. 1997). Using the newly cloned PLD1 and PLD2 proteins epitope-tagged with the influenza haemagluttinin (HA) protein, the localisation of these enzymes in rat embryo fibroblasts was investigated by Colley *et al.* (Colley, W. C., et al. 1997). PLD1 was reported to be present exclusively on the peri-nuclear region of the cell, on the

golgi, ER and late endosomes but not in the cytoplasm or on the plasma membrane in quiescent or serum-stimulated cells (Colley, W. C., et al. 1997). However Brown *et al.* report that PLD1b localised to secretory granules and lysosomes and not the golgi (Brown, F. D., et al. 1998). The PLD1b enzyme was expressed as a fusion protein with green fluorescent protein in COS 1 and RBL-2H3 cells and was found to translocate from the secretory granules etc. to the plasma membrane upon stimulation with agonist and phorbol ester (Brown, F. D., et al. 1998). Colley *et al.* also reported that PLD2 was predominantly associated with the plasma membrane in quiescent cells and redistributed to submembranous vesicles in serum-stimulated cells. (Colley, W. C., et al. 1997).

1.4.3 Regulation of PLD by small G proteins

The activation of PLD by GTPγS in permeabilized HL-60 cells was reported to decline with time as cytosolic proteins leaked from the cells (Geny, B. and S. Cockcroft 1992, Geny, B., et al. 1993). This activity could be restored by addition of a factor within brain cytosol found to be ~16 kDa. This factor was purified to homogeneity by two groups and shown to be the small G protein ARF (Brown, H. A., et al. 1993, Cockcroft, S., et al. 1994). ARF was originally isolated by Kahn and Gilman as a protein which was essential for the ADP-ribosylation of $G\alpha_s$ by cholera toxin (Kahn, R. A. and A. G. Gilman 1984). The advent of the molecular cloning of mammalian PLD1 has shown that ARF is an activator of PLD (Hammond, S. M., et al. 1995, Hammond, S. M., et al. 1997, Park, S.-K., et al. 1997).

The translocation of ARF from the cytosol to membranes in response to agonist has been reported (Cavenagh, M. M., et al. 1996, Houle, M. G., et

al. 1995, Rümenapp, U., et al. 1995). Furthermore this translocation is associated with activation of PLD in HL-60 cells (Houle, M. G., et al. 1995). The translocation and association of ARF1, 3, 4 and 5 to golgi membranes was inhibited by brefeldin A (BFA) (Cavenagh, M. M., et al. 1996). BFA is an inhibitor of guanine nucleotide-exchange on ARF (Donaldson, J., et al. 1992, Helms, J. B. and J. E. Rothman 1992, Randazzo, P. A., et al. 1993). ARNO an exchange factor with a Sec-7 homology domain (Sec-7 is a protein from yeast that is involved in secretion) promotes guanine-exchange on ARF (Chardin, P., et al. 1996, Morinaga, N., et al. 1996). However ARNO is insensitive to BFA as is another factor isolated from spleen (Strader, C. D., et al. 1995), although a large BFA-sensitive ARF-guanine exchange protein has recently been cloned and described (Morinaga, N., et al. 1997).

ARF proteins are required for the binding of clathrin AP-1 and AP-2 adaptor proteins to golgi membranes; these proteins being components of pathway that controls endocytosis and transport from the trans-Golgi network to prelysosomal/endosomal compartments (Stamnes, M. A. and J. E. Rothman 1993, Traub, L. M., et al. 1993, West, M. A., et al. 1997, Zhu, Y., et al. 1998). Furthermore ARF proteins are necessary for the binding of the non-clathrin coatomer protein complex to golgi membranes and the formation of coatomer-coated vesicles which play a part in the trafficking of proteins from the ER to the golgi (Donaldson, J. G., et al. 1992, Palmer, D. J., et al. 1993, Serafini, T., et al. 1991). Brefeldin A is reported to block secretion (Fujiwara, T., et al. 1988, Lippincott-Schwartz, J., et al. 1989, Misumi, Y., et al. 1986), and also prevents the assembly of coatomer-coated vesicles on golgi (Orci, L., et al. 1991). Thus ARF is proposed to be involved in regulation of the secretory pathway. PLD has been found on

the golgi membrane and its activation by ARF is inhibited by BFA (Ktistakis, N. T., et al. 1995). Furthermore Ktistakis *et al.* suggest that PLD may mediate ARF-dependent formation of coatomer-coated golgi vesicles (Ktistakis, N. T., et al. 1996).

The involvement of Rho in the activation of PLD was first described in neutrophil lysates by Bowman *et al.* (Bowman, E. P., et al. 1993). The stimulation of PLD by Rho in other tissues has been reported (Kwak, J. Y., et al. 1995, Malcolm, K. C., et al. 1996, Malcolm, K. C., et al. 1994, Ohguchi, K., et al. 1995, Ohguchi, K., et al. 1996). Moreover the stimulation of PLD in HL-60 cell membranes by RhoA reported by Ohguchi *et al.* was disputed by Martin *et al.* who propose that only endogenous ARF activates PLD in these cells (Martin, A., et al. 1996, Ohguchi, K., et al. 1995, Ohguchi, K., et al. 1996). Similarly Park *et al.* reported that constitutively active ARF but not RhoA activated endogenous PLD in COS 7 cells, and that constitutively active RhoA but not ARF activated expressed rPLD1 (Park, S.-K., et al. 1997). Work done by Hammond *et al.* showed that RhoA, Rac1 and Cdc42 all activated hPLD1 to a similar extent, that was not additive (Hammond, S. M., et al. 1995, Hammond, S. M., et al. 1997). Synergism between ARF and Rho proteins in the activation of hPLD1 was also reported (Hammond, S. M., et al. 1997), in agreement with previous reports on partially purified PLD activities (Kuribara, H., et al. 1995, Siddiqi, A. R., et al. 1995, Singer, W. D., et al. 1995, Singer, W. D., et al. 1996). A 50 kDa factor partially purified appears to be enhance the activation of PLD by GTPγS activation of Rho (Kwak, J. Y., et al. 1995, Malcolm, K. C., et al. 1994, Ohguchi, K., et al. 1995). In agreement with these reports Vinggaard and colleagues, report the loss of RhoA but not ARF activation of PLD after partial purification of the enzyme from

human placenta (Vinggaard, A. M., et al. 1997). This unknown 50 kDa factor is uncharacterized at present. Rho is a regulator of the actin cytoskeleton and controls the production of stress fibres and focal adhesions in cells (Chrzanowska-Wodnicka, M. and K. Burridge 1996, Ridley, A. J. and A. Hall 1992). PA has been shown to stimulate actin polymerisation in IIC9 cells (Ha, K.-S. and J. H. Exton 1993) , moreover PLD has been implicated in the formation of stress fibres in porcine aortic endothelial (PAE) cells in a Rho-dependent manner (Cross, M. J., et al. 1996). Rho like ARF, also regulates the activity of phosphatidylinositol 4-phosphate 5-kinase (PIP-5K), the enzyme that is involved in the production of PIP_2 (Chong, L. D., et al. 1994), PA also stimulates this enzyme (Moritz, A., et al. 1992). Thus the rearrangement of the cytoskeleton ascribed to Rho activity may be due to the involvement of PLD.

The stimulation of PLD by the tyrosine kinase v-Src has been reported to involve the small G proteins Ras and RalA (Jiang, H., et al. 1995). Active PLD can be precipitated from cell lysates with immobilised GST-RalA fusion protein (Jiang, H., et al. 1995). The immunoprecipitated PLD activity is PLD1 and the interaction is a direct one (Luo, J. Q., et al. 1997). Further work by Luo and colleagues has shown that the direct interaction of RalA with PLD does not effect the PLD activity and that ARF is required and directly involved in an active complex with RalA and PLD1 (Luo, J., et al. 1998). Thus ARF, RalA and PLD may interact in a ternary complex when stimulated through a heptahelical receptor.

1.4.4 Regulation of PLD by Protein Kinase C

There is much evidence that the activation of PLD by PKC involves the initial activation of PLC and hydrolysis of PIP_2, since many agonists that activate PLD are also activators of PLC (Bocckino, S. B. and J. H. Exton 1996, Singer, W. D., et al. 1997, Yeo, E.-J. and J. H. Exton 1995, Yeo, E.-J., et al. 1994, Zheng, L., et al. 1994). The subsequent hydrolysis of PIP_2 by PLC generates inositol 3,4,5 trisphosphate (IP_3) and diacylglycerol (DAG), an activator of PKC. Inhibition of PKC using H7, staurosporine, chelerythrine, calphostin C and bisindolylmaleimide, or downregulation of PKC enzymes by prolonged treatment of cells with phorbol esters point to a partial or complete dependence of agonist regulation of PLD on PKC in many cell types. For example the downregulation of PKC enzymes has been shown to completely block the ability of agonists such as carbachol, bradykinin, or gonadotropin-releasing hormone (GnRH) to activate PLD (Liscovitch, M. and A. Amsterdam 1989, Martin, T. W., et al. 1989, Martinson, E. A., et al. 1989, van Blitterswijk, W. J., et al. 1991, van Blitterswijk, W. J., et al. 1991). Singer *et al.* first identified that PKC-α was the factor in porcine brain that activated PLD (Singer, W. D., et al. 1995, Singer, W. D., et al. 1996). The activation of PLD by PKC has been reported to be independent of phosphorylation and suggested to represent a protein to protein docking role (Conricode, K. M., et al. 1992, Conricode, K. M., et al. 1994, Ohguchi, K., et al. 1996, Singer, W. D., et al. 1996). The activation of the cloned hPLD1a, 1b and rPLD1 isoforms by PKC-α was phosphorylation-independent (Hammond, S. M., et al. 1997, Park, S.-K., et al. 1997). Although the activation of hPLD1 with PKC-α was synergistic with ARF and Rho proteins, there was no synergy between ARF, Rho and PKC-α in the activation of rPLD1 (Park, S.-K., et al. 1997).

1.4.5 Regulation of PLD by tyrosine kinases

Growth factors receptors such as those for platelet-derived growth factor and epidermal growth factor with intrinsic tyrosine kinase activity, are able to activate PLD (Kaskin, M., et al. 1992, Plevin, R., et al. 1991, Yeo, E.-J., et al. 1994). Also as noted in section 1.4.3, PLD activity was stimulated by v-Src transformation of cells in a Ras-RalA and ARF-dependent fashion. A role for a tyrosine kinase(s) in the activation of PLD by G protein coupled receptors has been suggested. Inhibitors of tyrosine kinase activity have been reported to block thrombin-stimulated PLD activity in platelets and carbachol-evoked PLD activity in HEK cells expressing the M_3 receptor (Schmidt, M., et al. 1994).

1.4.6 Physiological implications of PLD activation

The hydrolysis of PtdCho by PLD is proposed to provide the choline precursor for the synthesis of the neurotransmitter acetylcholine (ACh) in human neuronal (LA-N-2) cells (Lee, C. H., et al. 1993). The levels of ACh are reduced in tissue taken from patients with Alzheimer's disease, along with the activity of PLD and choline acetyl-transferase (an enzyme responsible for acetylcholine formation) (Kanfer, J. N., et al. 1986). Thereby implicating PLD in the production of ACh in vivo, and the pathophysiology of this disease.

The induction of the transcription factor AP-1 is proposed to be downstream of PLD activation in human T lymphoid Jurkat cells. The involvement of PLD in the activation of this factor is suggested by the co-activation of both PLD and AP-1 in Jurkat cells. Furthermore the activation of AP-1 was blocked by the presence of ethanol, and propanolol (an inhibitor of PPH) (Mollinedo, F., et al. 1994). In addition, AP-1 was stimulated after the addition of PA (Mollinedo, F., et al. 1994). The

activation of PLD by platelet-activating factor (PAF) and GnRH is reported to regulate the expression of the immediate early response genes c-fos and c-jun, which are components of the AP-1 transcription factor (Bohmann, D. and R. Tijan 1989, Cesnjaj, M., et al. 1995, Liu, B., et al. 1997).

The production of PA is implicated in the regulation of the respiratory burst in neutrophils, which results in the release of reactive oxygen species that kill bacteria and mediate the inflammatory response. PA has been linked to the stimulation of NADPH oxidase (the enzyme that catalyses the production of superoxide (O_2^-) species), and this activation was shown to be inhibited in the presence of primary alcohols such as ethanol and butanol (Bauldry, S. A., et al. 1991, Bonser, R. W., et al. 1989, Rossi, F., et al. 1990).

The production of PA and its rapid dephosphorylation by phosphatidate phosphohydrolase to generate DAG produces a potential activator of PKC. It is not clear however that the side chain composition of the diglycerides (Hodgkin, M. N., et al. 1998, Pettitt, T. R., et al. 1997) and perhaps also its compartmentalisation are ideal for this role. Nevertheless the activation of PKC by agonist stimulation is commonly reported to be biphasic, with a rapid activation of PKC that is transient and a longer term stimulation of PKC. This profile of PKC activation has been proposed to reflect the differential production of DAG by PLC and PLD activities. PLD is suggested to be responsible for the prolonged formation of DAG and activation of PKC (Exton, J. H. 1994). Some reports indicated that PC-derived DAG was ineffective in activating PKC (Kiley, S. C., et al. 1991, Martin, T. W., et al. 1989), however work done in IIC9 fibroblasts by Ha *et al.* showed that PtdCho-derived DAG translocated PKC-ε to the membrane in a sustained manner (Ha, K.-S. and J. H. Exton 1993). The

PIP_2 breakdown evoked by thrombin in IIC9 cells caused only a transient translocation of PKC-α, whilst the second peak of DAG derived from PtdCho breakdown was associated with a sustained membrane association of PKC-ε, which is a calcium-independent isozyme, thus there was a PKC isozyme-specific translocation of PKC enzymes (Ha, K.-S. and J. H. Exton 1993).

Experiments carried out *in vitro* have suggested that PA is a regulator of numerous proteins involved in signal transduction, although the hydrolysis of PA by a PA-specific PLA_1 or PLA_2 (Higgs, H. N. and J. A. Glomset 1996, Higgs, H. N., et al. 1998, Thomson, F. J. and M. A. Clark 1995), thereby producing lysoPA may complicate this view. PA has been proposed as an activator of PKC-ζ (Limatola, C., et al. 1994, Nakanishi, H. and J. H. Exton 1992) and other protein kinases (Bocckino, S. B., et al. 1991, Khan, W. A., et al. 1994, McPhail, L., et al. 1995). Also PA has been suggested as an stimulator of a protein tyrosine phosphatase (Tomic, S., et al. 1995, Zhao, Z., et al. 1993), PtdIns-PLC-γ (Jacowski, S. and C. O. Rock 1989, Jones, G. A. and G. Carpenter 1993) and PIP-5K (Moritz, A., et al. 1992). In addition PA has been reported to regulate the GTPase activating proteins (GAPs) for small G proteins e.g. the stimulation of n-chimaerin, an activator of Rac GAP (Ahmed, S., et al. 1993) and the inhibition of Ras GAP (Tsai, M., et al. 1989). PA is also reported to bind to Raf-1 kinase (Ghosh, S., et al. 1996). PA has been observed to stimulate arachidonic acid release from fibroblasts implying activation of PLA_2 (Murayama, T. and M. Ui 1987) although a similar effect is seen with lysoPA which acts as agonist at its own GPCR (van Corven, E. J., et al. 1989). Moreover the stimulation of PtdCho-PLD by PA or lysoPA has also been reported (Ha, K.-S. and J. H. Exton 1993, Ha, K.-S. and J. H. Exton 1994, van der Bend, R.

L., et al. 1992), suggesting a positive feedback loop. LysoPA is a potent mitogen (Jalink, K., et al. 1994), and its mitogenic effect is inhibited by pertussis toxin, indicative of a $G\alpha_{i/o}$-mediated mechanism. The cloning of cDNAs for a LysoPA-specific receptor has been reported, and the receptor couples to $G_{i/o}$ and G_q (An, S., et al. 1998, Dickens, A. S., et al. 1997, Guo, Z., et al. 1996, Hecht, J. H., et al. 1996). It is likely that the majority of the mitogenic effects of LysoPA involve the Ras/mitogen-activated protein (MAP) kinase pathway, as suggested by numerous reports (Hordijk, P. L., et al. 1994, Howe, L. R. and C. J. Marshall 1993, Kumagai, N., et al. 1993, McLees, A., et al. 1995, Moolenaar, W. H., et al. 1997). In other systems however, the GnRH and PAF-induced activation of c-fos and c-jun synthesis may depend on a PLD-mediated pathway (Cesnjaj, M., et al. 1995, Liu, J., et al. 1995).

There is a great deal of data suggesting that PLD is a major regulatory component of protein secretion and transport. The cloned hPLD1 enzyme was found to localise to secretory granules in human neutrophils, COS 1 and RBL-2H3 cells and upon stimulation with agonist it translocates to the plasma membrane, implicating hPLD1 in protein exocytosis (Brown, F. D., et al. 1998, Morgan, C. P., et al. 1997). In agreement with this, Caumont *et al.* reported that ARF6 translocated from secretory granules to the plasma membrane in stimulated chromaffin cells and this was associated with an increase in PLD activity and the secretion of catecholamines. The use of a synthetic peptide to block the interaction of ARF6 with its target proteins blocked both the activation of PLD and the secretory response. (Caumont, A.-S., et al. 1998). Purified PLD directly stimulates the release of secretory vesicles from the trans-golgi network in an ARF-dependent manner (Chen, Y. G., et al.

1997). The stimulation of the PIP-5K activity by PA (Moritz, A., et al. 1992), and the activation of PLD isoforms by PIP_2 (Hammond, S. M., et al. 1995, Hammond, S. M., et al. 1997, Park, S.-K., et al. 1997) lead to the proposal that PLD activities on secretory vesicles or at the donor and target membrane for vesicle transport, would produce PIP_2 and PA which would both maintain PLD activity and allow the fusion of vesicle and target membranes, as PIP_2 and PA are fusogenic lipids (Eastman, S. J., et al. 1992, Liscovitch, M. and L. C. Cantley 1995).

Therefore with this background, the present study aimed to address the characteristics of the activation of PLD by class I and II GPCRs. The role of the small G proteins ARF and Rho in the activation of PLD was investigated, and the regions involved in the control of receptor coupling to PLD were studied.

Chapter 2

Materials and Methods

2.1 MATERIALS

Tissue culture media

Minimum essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), Medium 199, Earle's balanced salt solution (EBSS), Hank's Buffered Saline Solution (HBSS) without calcium, magnesium or phenol red, Ham's nutrient mixture F12, OptiMem, dimethylsulphoxide (DMSO), newborn calf serum (NCS), ampicillin, tetracycline, penicillin, streptomycin, geniticin (G418), trypsin, phosphate buffered saline (PBS), 80cm^2 and 175 cm^2 Nunclon flasks were all obtained from Gibco Life Technologies, Paisley, Scotland U.K. Foetal calf serum was purchased from Sera Lab, Crawley Down, Sussex, U.K. DEAE dextran was purchased from Promega UK Ltd., Southampton, U.K. All other tissue culture plasticware was purchased from Costar UK Ltd., Bucks, U.K. or Greiner Laboritechnik Ltd., U.K.

Animals

COB-Wistar rats were obtained from Charles River U.K. Ltd., Margate, Kent, U.K., or were obtained from a colony bred in this department which were derived from Charles River Wistar rats.

Radiochemicals

[9,10-^{3}H]palmitate (40 Ci/mmol), [^{3}H]N-methyl scopolamine (85 Ci/mmol), [^{125}I]PACAP-27 (2200Ci/mmol) were obtained from NEN DuPont, Germany, Na ^{125}I (400 mCi/mmol from ICN radiochemicals, Irvine, CA, U.S.A. and *myo*-[2-^{3}H]inositol from Amersham International plc, Aylesbury, Bucks, U.K.

Agonists and Ligands

Gonadotropin-releasing hormone, angiotensin II, carbachol (carbamylcholine chloride), histamine ((2-[4-imidazolyl]ethylamine), thrombin, bradykinin, 5-hydroxytryptamine ((3-(2-aminoethyl)-5-hydroxyindole; serotonin), sodium fluoride (NaF), beryllium chloride ($BeCl_2$), GTPγS (guanosine 5′-O-(3-thiotriphosphate)), ionomycin (free acid, *Streptomyces conglobatus*), and forskolin (*Coleus forskohlii* (7β-acetoxy-8,β-epoxy-1α,6β,9α-trihydroxy-labd-14-en-11-one) were all purchased from Sigma-Aldrich Chemical Company, Poole, Dorset, U.K., U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$) was purchased from Affiniti Research Products Ltd., Mamhead Castle, Exeter, U.K. Vasoactive Intestinal Peptide (VIP), pituitary adenylate-cyclase activating polypeptide-38 (PACAP-38) and helodermin were purchased from Calbiochem-Novabiochem (U.K.) Ltd., Nottingham, U.K. Ala-pFPhe-Arg-Cha-HArg-Tyr-NH_2 (TRP) was purchased from Neosystem Laboratoire, Croydon, Surrey, U.K. $GPPCH_2P$ (guanylyl-(β,γ-methylene)-diphosphate) was obtained from Biomol Ltd., Cheshire U.K. Phosphatidylbutanol was obtained from Avanti Polar Lipids Inc., Alabaster, Alabama, U.S.A.

Biochemicals

Brefeldin A (from *Penicillium brefeldianum*, γ,4-dihydroxy-2-[6-hydroxy-1-heptenyl]-4-cyclopentanecrotonic acid λ lactone), 2′-O-monosuccinyl-adenosine 3′: 5′-cyclic monophosphate tyrosyl methyl ester, Tween-20 (Polyoxyethylenesorbitan monolaurate), polyethylene glycol, isobutylmethylxanthine (IBMX), aprotinin, protein G Sepharose CL-4B, leupeptin, bacitracin, soybean trypsin inhibitor, pepstatin A, sodium

orthovanadate (Na_3VO_4), cholera toxin (*Vibrio cholerae*), pertussis toxin (*Bordatella pertussis*), β-mercaptoethanol, phorbol 12,13-dibutyrate, monensin, monodansylcadaverine, N-methyl atropine, HEPES (4-(20 hydroxyethyl)-1-piperazine ethanesulfonic acid), bovine serum albumin (BSA: essentially fatty acid free and fraction V), Phosphatidylcholine and phosphatidylserine were obtained from Sigma-Aldrich Chemical Company, Poole, Dorset, U.K. U73122, {1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione}, C3 exoenzyme (from *Clostridium botulinum*) and GF109203X (bisindolylmaleimide I; 2-[1-3(-dimethylaminopropyl)-1H-indol-3-yl]3-(1H-indol-3-yl)-maleimide) were purchased from Calbiochem-Novabiochem (U.K.) Ltd., Nottingham, U.K. AEBSF (4-(2-aminoethyl)-benzenesulphonyl fluoride) was purchased from Alexis Corporation (U.K.) Ltd., Nottingham U.K. Pfu (*Pyrococcus furiosus*) DNA polymerase were obtained from Stratagene Ltd., Cambridge, U.K. *Hinc*II restriction enzyme and T_4 DNA ligase were obtained from Promega UK Ltd., Southampton, U.K.

Immunological reagents

Sheep polyclonal anti-ARF1/3 immunoglobulin (antigen $ARF1_{98-112}$), was a gift from M. J. O. Wakelam, Institute for Cancer Studies, Birmingham University Medical School, Birmingham, U.K (Martin, A., et al. 1996). Rabbit polyclonal anti-M_3 receptor serum (antigen M_3 $receptor_{561-578}$) was a gift from B. B. Wolfe, Department of Pharmacology, Georgetown University School of Medicine, Washington, D. C. U.S.A. (Wall, S. J., et al. 1991). Rabbit polyclonal anti-RhoA IgG (antigen $RhoA_{119-132}$), rabbit monoclonal anti-RhoA IgG (clone 26C4; antigen $RhoA_{120-150}$) and rabbit polyclonal anti-AT_1 receptor IgG (antigen AT_1 $receptor_{15-24}$) were obtained from Santa Cruz Biotechnology, Santa Cruz,

Ca, U.S.A. Mouse monoclonal anti-ARF IgG (clone 26) and mouse monoclonal anti-Ras IgG (antigen entire $p21^{ras}$) were obtained from Transduction Laboratories, Affiniti Research Products, Mamhead Exeter, U.K. Mouse monoclonal anti-ERK1/2 (antigen $ERK1_{325-345}$) was obtained from Zymed Laboratories, Cambridge BioScience, Cambridge, U.K. Horseradish peroxidase-conjugated, donkey anti-rabbit and sheep anti-mouse secondary antibodies and non-immune rabbit and sheep serum were obtained from the Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Lanarkshire, U.K.

Plasmids

The plasmid pcDNA1/AMP was obtained from InVitrogen, R&D Systems Europe Ltd., Abingdon, U.K. pcDNA1/AMP plasmids carrying the wild-type, Asn318, Asp87-Asn318 mutant GnRH, and wild-type, Asp376 mutant 5-HT2A receptors were a gift from S. C. Sealfon, Fishberg Center for Neurobiology and Department of Neurology, Mount Sinai School of Medicine, New York, New York, U.S.A. (Sealfon, S. C., et al. 1995, Zhou, W. C., et al. 1994). pBluescript was obtained from Stratagene Ltd., Cambridge, U.K. The pCMV5 construct carrying wild-type and a dominant negative construct of RhoA were a gift from G. M. Bokoch, Department of Immunology and Cell Biology, Scripps Research Institute, La Jolla, CA. U.S.A. (Zhang, S., et al. 1995).

Oligonucleotide Primers

cDNA primers which spanned the junction site for the construction of the VIP_2-PACAP receptor chimaeric receptors, corresponding to the coding region for TMD7 were synthesised by Cruachem Ltd., Glasgow, U.K., or Oswel DNA Service, Southampton, U.K.

PACAP: 5′ GTCGCAGTTCTATATATTGCTTCTTGAATGGGGAGGT

VIP_2: 5′ AAGCAATATAAGAACTGCGACC

General reagents

Bicinchoninic acid and Coomassie protein determination kits were purchased from Pierce U.K. Ltd., Cambridge, U.K. The plasmid maxi purification kit was obtained from Qiagen Ltd., Crawley, West Sussex, U.K. The Wizard cDNA purification system was purchased from Promega UK Ltd., Southampton, U.K. Bond Elut and Brownlee chromatography columns were obtained from Anachem Ltd., Luton, Beds. LK5D silica gel thin layer chromatography plates were obtained from Whatman International Ltd., Kent, U.K. Immobilon (polyvinyldifluoride) membranes were purchased from Millipore, Watford, U.K. Enhanced Chemi-Luminescence (ECL) detection system, Hypam and Phenisol photographic products were obtained from Amersham International plc, Ayelsbury, Bucks., U.K. Standard laboratory reagents of Analar grade and Chromacol glass vials were obtained from BDH/Merck Ltd., Poole, Dorset, U.K.

2.2 METHODS

2.2.1 Cell culture

Cell lines were grown and maintained under a humidified atmosphere of 95% O_2: 5% CO_2 at 37°C with fresh medium added every 3-4 days. The cells were harvested by either trypsin digestion or a brief incubation with Hank's Buffered Saline Solution (HBSS) without calcium, magnesium or

phenol red containing 0.1% (w/v) EDTA. The cell medium was removed and 0.25% trypsin (v/v) solution in Gibco solution A (0.4 g/l KCl, 2.2 g/l $NaHCO_3$, 6.8 g/l NaCl, 1.0 g/l glucose, 0.005 g/l Phenol Red) was applied (1 ml per 80 cm^2 flask or 2 mls per 175 cm^2 flask) to the cell layer and then aspirated after approximately 15 seconds. After a further 10 minutes the flask was agitated to dislodge cells and then they were washed off the flask's surface with medium and pelleted by centrifugation at 200*g* for 5 minutes. The cells were then resuspended in medium and distributed into new flasks (usually at a ratio of 1:3 or 1:4) or seeded into 12- or 24-well plates for assay. Alternatively the HBSS/EDTA solution was applied (1 ml per 80 cm^2 flask or 2 mls per 175 cm^2 flask) for 10 minutes before the cells were washed off as described above. All cell lines were maintained in 75 cm^2 flasks (Costar Bucks, U.K.) unless otherwise stated.

For storage purposes, 0.5 ml volumes of cells at 10^6 cells/ml in medium or Foetal Calf Serum containing 7-10% dimethylsulphoxide (v/v) (DMSO) were aliquoted into cryosafe tubes and placed at -70°C overnight in a polystyrene box. The tubes were then placed into liquid nitrogen. To recover the cells from the liquid nitrogen the tubes were allowed to thaw at room temperature and then warmed in a 37°C water bath for several minutes. A small volume of medium was added to the tube and the cells were removed and resuspended in approx. 8 mls of appropriate medium. The cells were pelleted by centrifugation at 200*g* for 5 min before being resuspended in medium and transferred into a 25 cm^2 flask. The next day the medium was aspirated off and fresh medium added to remove traces of DMSO. The cells were then grown as normal

COS 7: Green Monkey kidney fibroblast cell line

This monkey kidney fibroblast cell line was a gift from Janet Allen (Glasgow University, Scotland, U.K.). These cells were maintained in Dulbecco's Modified Eagle Medium (high glucose with 580 mg/l l-glutamine, 4500 mg/l D-glucose, without sodium pyruvate) supplemented with 10% newborn calf serum (v/v) and 100 units/ml penicillin and 100 µg/ml streptomycin and were passaged every 5 to 7 days when close to 100% confluency. These cells were used for signal transduction assays using transiently transfected exogenous receptors. The cDNAs for the chimeric VIP2/PACAPshort and VIP2/PACAPlong (VP/4/7b/2.1c and VP/4/7/2.1c, pCDNA1) receptors were transfected into COS 7 cells using DEAE dextran as described below. The COS 7 cell line was maintained in 175 cm^2 flasks (Nunclon).

Chinese Hamster Ovary (CHO) cells

CHO cells, used to stably expressing transfected receptor cDNA (VIP_1, VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors), were grown and maintained in Nutrient Mixture Ham's F12 medium supplemented with 10% NCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 300 µg/ml geniticin (G418). Geniticin was present to allow selection of cells stably expressing transfected DNA. Cells were passaged every 6-7 days when 80-90% confluent.

1321N1 Human astrocytoma cells

1321N1 cells were maintained in DMEM supplemented with 10% NCS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were passaged every 6-7 days when 100% confluent.

2.2.2 Preparation of cDNA

Transformation of competent cells

Approx. 100 ng of plasmid cDNA was added to a 50 µl aliquot of MC1061/P3 competent cells (a strain of Escherichia coli bacteria, prepared in house) before being mixed gently and left on ice for 30 minutes. The cells were then heated for 45 seconds at 42°C and placed back on ice for 10 minutes. The cells were added to 1 ml of Luria Bertani (LB)-broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) and placed on a rotating mixer (New Brunswick Scientific, Edison, N.J. U.S.A.) in a 37°C incubator for 1 hour. 100 µl of this suspension was spread onto LB-agar plates (LB-broth + 1% bactoagar) supplemented with ampicillin at 12.5 µg/ml (Sigma) and tetracycline at 7.5 µg/ml (Gibco) to select for plasmid-containing cells. The plates were left in the 37°C incubator overnight. A distinct colony was selected from the plate and used to inoculate a 2 ml volume of LB-broth containing ampicillin and tetracycline. The culture was incubated overnight at 37°C on a rotator. The 2 ml culture was then used to inoculate a 500 ml volume of L-broth containing ampicillin and tetracycline in a conical flask which was left in an orbital shaking incubator overnight at 37°C.

Plasmid purification

Plasmid purification was carried out using the Qiagen Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. The bacterial cells were harvested by centrifugation at 6000*g* for 15 min at 4°C (Sorvall GSA rotor). The supernatant was carefully decanted and the pellet resuspended in 10 ml of Buffer P1 (resuspension buffer: 50 mM Tris HCl, pH 8.0; 10 mM EDTA) containing 100 µg/ml RNase A solution. After resuspension, 10 ml of Buffer P2 (lysis buffer: 200 mM NaOH, 1% sodium

dodecyl sulphate (w/v)) was added and the solution mixed gently by inversion and left to incubate at room temperature for 5 min. The lysis reaction causes the production of a viscous solution. 10 ml of chilled Buffer P3 (neutralisation buffer: 3.0 M potassium acetate, pH 5.5) was added, the solution was immediately mixed by inversion and incubated on ice for 20 min. A viscous precipitate of genomic DNA, protein, cell debris and SDS is visible at this point. The solution was mixed again and loaded into polypropylene tubes for centrifugation in the Sorvall SS-34 rotor at 20,000*g* for 30 minutes at 4°C. The supernatant containing the plasmid DNA was rapidly removed and recentrifuged for 15 min at 20,000*g* to remove particulate matter and prevent overloading of purification columns. A Qiagen-tip 500 was equilibrated by the application of 10 ml of QBT buffer (equilibration buffer: 750 mM NaCl; 50 mM MOPS (3-[N-morpholino] propanesulfonic acid), pH 7.0; 15% isopropanol (v/v); 0.15% (v/v) Triton X-100). The supernatant was applied to the column and washed with 2 x 30 ml of buffer QC (wash buffer: 1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)). The DNA was eluted with 15 ml of buffer QF (elution buffer: 1.25 M NaCl; 50 mM Tris HCl, pH 8.5; 15% isopropanol (v/v)) and collected. The DNA was precipitated by the addition of 10.5 ml of isopropanol at room temperature, the solution was mixed and centrifuged at 15,000*g* for 30 min at 4°C (Sorvall SS-34 rotor). The outside of the tube was marked beforehand to identify the position of the pellet which can be difficult to see. The pellet was washed with 5 ml of room temperature 70% ethanol (v/v) and centrifuged at 15,000*g* for 10 min. The last step was repeated and then the pellet was air-dried for 10 minutes before being resuspended in TE buffer (10 mM Tris HCl, pH 8.0; 1 mM EDTA) pH 8.0, for use in transfection.

2.2.3 Transfection of recombinant DNA using DEAE dextran

COS 7 cells trypsinised or treated with EDTA as described earlier were plated out into 80 cm^2 flasks at approx. 50% confluency for transfection the following day. The growth medium was removed and the cells washed twice with prewarmed OptiMem for 10 min, before the transfecting medium was added to the cells. The transfecting medium consisted of the cDNA (pcDNA1 carrying receptor cDNA) in OptiMem (30 µg of cDNA/flask) and DEAE dextran at 0.8 mg/ml (DEAE dextran stock was at 10 mg/ml in PBS and filter-sterilised), 2 mls of this solution was added to each flask and gently distributed over the cell layer. After 30 minutes incubation, 3 mls of OptiMem containing 0.4 mg/ml DEAE dextran and 167 µM chloroquine phosphate (Sigma) (10 mM stock made up in PBS and filter-sterilised) was added to the solution and the flasks were left to incubate for a further 3 hours. After this time the transfecting medium was aspirated off and replaced with PBS containing 10% DMSO for 2 minutes. This medium was then aspirated and replaced with the growth medium for transfected COS 7 cells: DMEM supplemented with 2% UltroSer G (v/v) (Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin. 24 hours later the cells were trypsinised or treated with EDTA and seeded into 24- or 12-well plates for assay (2 x 80 cm^2 flasks per plate). Alternatively for binding studies the medium in the flasks was changed to DMEM with 10% foetal calf serum. The cells were assayed after a further 40 hours

2.2.4 Production of CHO cell clones stably expressing the VIP or PACAP receptors (by Dr Eve Lutz and Christine Morrison)

The cDNAs encoding the VIP_1, VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptor were introduced into the expression vector pCDNA1, containing the neomycin resistance gene, the resulting recombinant constructs were termed PV19, R4, R7b and R7. Chinese hamster ovary (CHO) cells were transfected with the receptor plasmids by lipofection. 24 hours after transfection, cells were trypsinised and plated into (Greiner) cloning plates. 48 hours after transfection, geniticin-resistant cells were selected by incubating in Ham's F12 nutrient media containing 10% newborn calf serum (v/v, NCS), and 100 units/ml penicillin, 100 µg/ml streptomycin, and supplemented with 500 mg/ml geneticin. After one month of growing cells in geneticin-containing media, colonies were selected and replated into 24 well plates, and maintained in the selection media. Clonal lines expressing the different receptors were selected for their ability to stimulate adenylate cyclase in response to VIP, PACAP and other VIP-like peptides, using a cAMP radioimmunoassay. The expression of the mRNA for the various receptors was also shown by Northern analysis.

2.2.5 Liposome treatment and transfection of 1321N1 cells

1321N1 cells were seeded into 12-well plates, coated with fibronectin (5 $\mu g/cm^2$). When 60-80% confluent the cells were treated with liposomes prepared with 2.6 µl Lipofectamine reagent (Life Technologies/Gibco) in 420 µl OptiMem. Cells were transfected with liposomes carrying either *Clostridium botulinum* ADP-ribosyltransferase C3 (2 µg/well; 5 hours), or CMV-RhoA constructs (0.5 µg DNA/well; 7 hours) or no additions. Rho-construct transfected cells were returned to the normal growth medium for 48 hours, prior to quiescing and labelling of cells for assay.

2.2.6 Assay of Phospholipase D activity

Measurement in clonal cell lines

All cell lines to be used in a PLD assay were first plated into 12 well dishes (Costar U.K.). Prior to assay, cells were quiesced for 18 hours in serum-free medium and the fatty acid moiety of phosphatidylcholine was labelled for 18 hours with 10 μCi/ml [9,10-^{3}H] palmitate (40 Ci/mmol, Du Pont). The activation of PLD normally leads to the production of phosphatidic acid and choline. In the presence of a primary alcohol, PLD catalyses the production of a phosphatidylalcohol by the transfer of the phosphatidyl moiety of a phospholipid to the alcohol. PLD activity was thus monitored as the production of [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut)when cells were stimulated in the presence of 30 mM butan-1-ol. The morning of the assay the labelling medium was removed and the cells were washed twice with Minimum Essential Medium (MEM, Gibco Life Technologies) + 1% Bovine Serum Albumin (w/v) (BSA, Sigma, essentially fatty acid free), before being incubated with 0.5 ml MEM + 0.5% BSA (w/v). The assay was started with addition of agonist, all agonist and drug treatments of cells was for 30 mins at 37°C in a humidified atmosphere of 95% O_2: 5% CO_2 unless otherwise stated. The assay was terminated by aspiration of the assay medium and addition of 0.5 ml ice-cold methanol. Cells were homogenised and the extraction of the [^{3}H]PtdBut product followed a method of lipid extraction described previously (Bligh, E. G. and W. J. Dyer 1959). Samples transferred to 2 ml glass vials (Chromacol, BDH), 500 μl chloroform and 400 μl H_20 were added to give a ratio of methanol/chloroform/H_20 of 1:1:0.8. Samples were vortexed and left for 15 mins, then spun for 8 mins in a centrifugal evaporator (Gyrovap, V. A. Howe, Banbury, U.K.) to separate the aqueous

and organic layers. The upper aqueous layer was removed and an aliquot or all of the lower organic phase was evaporated under vacuum at 30°C in the centrifugal evaporator for 30-50 min. The extract was resuspended in 50 µl of a mix of chloroform and methanol in the ratios of 19:1. The sample was applied to a LK5D, silica gel plate (Whatman). The plates were developed using the organic phase of the mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water, in the ratios of 110:50:20:100. The region of the TLC plate corresponding to [^{3}H]PtdBut was identified using iodine staining of phosphatidylbutanol standards (Avanti Polar Lipids, U.S.A.), or a [^{14}C]PtdBut standard (gift from R. Randall, Wellcome Research Laboratories, Kent, U.K.). Also the agonist or phorbol ester-stimulated [^{3}H]PtdBut peak was lost in the absence of butan-1-ol. This region was scraped into vials (Canberra Packard). 4 ml of scintillant (Emulsifier-Safe LSC cocktail, Packard) was added to each vial, samples then shaken briefly and counted on the Beckman LS 5801 Series analyzer (Beckman).

Measurement in pituitary tissue

Adult male COB Wistar rats (250 g) were maintained under controlled lighting (lights on from 05.00 to 19.00 hours) and temperature (22°C) with free access to food pellets (CRM, Labsure, Manea, Cambs, U.K.) and tap water. Rats were decapitated and the anterior pituitaries removed and quartered then distributed into 2 conical flasks and incubated in warmed MEM at 37°C under an atmosphere of 95% O_2: 5% CO_2 to recover. The tissue was washed twice with fresh MEM before tissue equivalent to 4.5 pituitaries was placed in 2 ml MEM, and labelled for 2 hours with 30 µl of [9,10-^{3}H] palmitate (75 µCi/ml), whilst gently shaking. Pituitary tissue was then washed with MEM containing 1% BSA (w/v) to remove any

unincorporated label and pituitary quarters were distributed into assay vials containing 400 µl of MEM, plus 0.5% BSA (w/v). Stimulation of PLD with agonists in the presence of 30 mM butan-1-ol, was for 30 min as described above. The assay was terminated by the addition of 500 µl methanol and then chloroform. Tissue was homogenised and the [^{3}H]PtdBut was extracted as described above. The rest of the assay followed normal protocol.

Permeabilisation of 1321N1 cells and activation of PLD by GTP analogues

Confluent 1321N1 cells in fibronectinised 12-well plates were labelled and washed as described above. Cells were preincubated as required with 100 µM carbachol, for 10 min at 37°C. The preincubation medium was replaced with intracellular buffer (Rümenapp, U., et al. 1995) consisting of 6 µM digitonin, 135 KCl, 5 mM $NaHCO_3$, 5 mM EGTA, 5.6 mM glucose, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 20 mM HEPES, 2 mM ATP, 10 µM NAD, 6 µM digitonin, 30 mM butan-1-ol. GTP analogues were included as required; 100 µM GTPγS, 200 µM $GPPCH_2P$ or 10 mM NaF in the presence of 30 µM $BeCl_2$. Brefeldin A was used at a concentration of 100 µM and C3 exoenzyme was used at a concentration of 1 µg/ml. PLD assays were continued by the normal protocol.

2.2.7 Assay for [^{3}H]inositol phosphate production

Cells in 12-well tissue culture plates, maintained at 37°C in a 95% O_2 : 5% CO_2 environment, were labelled with 0.5 or 1 µCi/ml of *myo*-[2-^{3}H]inositol (Amersham) for 16-18 hours in Earle's Balanced Salt Solution with 10 mM glucose, 10 mM HEPES, pH 7.4 (NaOH). The cells were then washed twice in Earle's Balanced Salt Solution with 10 mM glucose, 10 mM HEPES, 0.2% bovine serum albumin (Fraction V; Sigma), pH 7.4

(NaOH) and preincubated for 10 minutes with 10 mM LiCl before agonist stimulation. Reactions were stopped by aspiration of medium and addition of 700 µl of ice-cold 1.34M trichloroacetic acid. The wells were scraped and the solution transferred into a 1.5 ml eppendorf tube for centrifugation to pellet the precipitated protein (5 minutes, 12,000*g*,, 4°C Jouan MR 1815 centrifuge, Jouan U.K.), a 500 µl sample of the supernatant was then added to 50 µl of 0.1 M ethylenediaminetetraacetic acid (EDTA) and 500 µl of a 1:1 mixture of 1,1,2-trichlorotrifluoroethane and tri-n-octylamine. The sample was vortexed and centrifuged (5 minutes, 12,000*g*,, 4°C) and a 300 µl volume of the aqueous phase was added to 200 µl of 1 M $NaHCO_3$ containing universal indicator. The sample was applied to a 1 ml column of Dowex anion exchange resin (1x8 resin, formate form, 200-400 mesh; Bio-Rad) and a stepwise gradient of ammonium formate was used to elute the [^{3}H]inositol phosphates, a method previously described by Berridge et al. (1983).

The sample in the column was washed with:

- 15 ml of UHP water
- 5 ml of 50 mM ammonium formate
- 10 ml of 1 M ammonium formate/0.1 M formic acid. This fraction contains the [^{3}H] inositol phosphates and was collected in a Zinsser scintillation vial (Zinsser Analytic GmbH)
- 5 ml of 2 M ammonium formate/0.1 M formic acid
- 15 ml of UHP water

500 µl aliquots were distributed into Pony vials (two from each collected fraction) and 4 ml of Emulsifier-Safe LSC cocktail (Packard) was added to each Pony vial. The samples then shaken briefly and left overnight before being counted by liquid scintillation counting, as described for the PLD assay .

2.2.8 Radioimmunoassay for cAMP production

Iodination of cAMP

2'-O-Monosuccinyladenosine 3': 5'-cyclic monophosphate tyrosyl methyl ester (Sigma U.K.) was radiolabelled with $Na^{125}I$ (Amersham International, Aylesbury, Buckinghamshire), using the chloramine-T method as described previously (Greenwood, F. C., et al. 1963). The labelled peptide was purified by reverse-phase liquid chromatography, using a 1 ml 'Bond Elut' column (Anachem Ltd., U.K.). The column was washed with a gradient of methanol/trifluoroacetic acid (TFA). Radiolabelled cAMP was eluted with 40-50% methanol/0.1%TFA (v/v). Specific activities of 3 mCi/µg were routinely obtained.

Cells were washed in MEM-BSA (0.25%) and pre-incubated with 0.5 mM isobutylmethylxanthine (IBMX) for 15 minutes before agonist stimulation. The stimulation period was terminated by either: aspiration of the medium and addition of a 500 µl volume of ice-cold 0.1M HCl (for intracellular cAMP measurement) or the addition of an equal volume of ice-cold 0.2M HCl to the medium (for total cAMP measurement). The plates were then frozen at -70°C. After thawing, the cells were homogenised by trituration. Duplicate 50 µl aliquots were taken from each well and assayed for cAMP content by radioimmunoassay.

All solutions were made up in 50 mM sodium acetate buffer (pH 6.0), 0.1% BSA (fraction V), 0.1% sodium azide (w/v) and the assays carried out in polypropylene microrack tubes (1.2 ml, Alpha Labs). RIB7 antiserum at a final concentration of 1:166,000 was used as a primary antibody, ^{125}I-cAMP at approx. 10,000 cpm per tube was added and the tubes were mixed by Vibrax and left at 4°C overnight. Donkey anti-rabbit IgG (at a titre of 1:400) was used as a secondary antibody and non-immune rabbit serum was also added at a titre of 1:40 for a final assay volume of 275 µl. The tubes were mixed and incubated at 4°C for 3 hours. 700 µl of ice-cold PEG-8000 (8.7%) in 0.1M sodium phosphate buffer (pH 7.7) was then added to each tube in order to aid the precipitation of the ^{125}I-cAMP/antibody complex and the tubes were spun at 1,500*g* for 25 minutes at 4°C. The supernatant was then aspirated off and the gamma radiation remaining in the pellet, and thereby the cAMP content, was determined using the Cobra Autogamma counter (Packard) and the associated software. The specific binding was typically 40% of the total applied counts and the non-specific binding typically comprised less than 10% of the specific binding. The assay included a cAMP standard curve running from 1 to 512 nM cAMP. The cAMP concentration of the unknowns were determined by interpolation of the % bound counts and the log of the cAMP concentration.

2.2.9 Solubilization and immunoprecipitation of M_3 receptors labelled by [^{3}H]N-methyl scopolamine ([^{3}H]NMS) and thrombin receptors labelled with [^{125}I]Ala-pFPhe-Arg-Cha-HArg-Tyr-NH_2 ([^{125}I]TRP)

TRP (Calbiochem-Navobiochem (U.K.) Ltd., Nottingham) was radiolabelled with Na^{125}I (Amersham International, Aylesbury, Bucks), using the chloramine-T method as described above. However labelled

TRP was purified by reverse-phase liquid chromatography, using a Brownlee 4.6 x 30 mm column (Anachem Ltd., U.K.) according to the method of Harmar and Rosie (Harmar, A. J. and R. Rosie 1984). Radiolabelled TRP was eluted with 70% methanol/0.1%TFA (v/v). Specific activities of 200 μCi/μg were routinely obtained.

1321N1 cells in 75 cm^2 tissue culture flasks (pre-incubated with carbachol (100 μM) or the thrombin agonist Ser-Phe-Leu-Arg-Asn-NH_2 (30 μM) for 10 min, were washed and homogenised in ice-cold buffer A with HEPES 20 mM, EDTA 3 mM NaOH pH 7.5, containing AEBSF 1 mM, aprotinin 2 μg/ml, leupeptin 4 μg/ml, pepstatin A 2 μg/ml, soybean trypsin inhibitor 50 μg/ml, NaF 1 mM and sodium orthovanadate 1 mM. The homogenate from the 1321N1 cells was spun at 12,000*g*, and the pellet/membrane fraction was used in the solubilisation procedure. Membranes were resuspended and solubilized in 15 volumes of 5 mM CHAPS, 0.1% Na cholate, 1M NaCl with the protease and phosphatase inhibitors used in the homogenisation step for 30 min at 4°C, whilst rolling (rotamixer, Stuart Scientific, Philip Harris Scientific, Coatbridge, U.K.). An equal volume of 20% (v/v) glycerol in CHAPS/cholate buffer without NaCl was added (with 0.6 mg/ml phosphatidyl choline, PtdCho, for M_3 receptors). 30 μl of a 1:1 dilution of protein G Sepharose CL-4B (Sigma), suspended in solubilization buffer was added to the solubilized sample and the sample was rolled for a further 20 min at 4°C, and then spun at 12,000*g* to remove proteins that bind non-specifically to protein G. The precleared sample was split between 2 ml tubes and incubated with sheep anti-ARF1/3 immunoglobulins (10-15 μl/ml), rabbit anti-RhoA IgG (2-3 μg/ml Santa Cruz Biotechnology), or non-immune IgG controls from sheep or rabbit (3 μg/ml) for 18 hours at 4°C on a tube roller. Antibody-

blocking control peptides were used in controls at 6 μg/ml. Antigen-antibody complexes were collected by addition of 50 μl/tube of the protein G-Sepharose suspension, incubation for 3 hours at 4°C, then spun at 12,000*g*.. 150 μl aliquots of beads and supernatant from the previous spin were assayed for solubilized receptor binding sites. M_3 receptors were assayed in 500 μl assays containing: 10 mM $MgCl_2$, 200 mM NaCl, 10% (v/v) glycerol, 3 mg/ml PtdCho, 20 mM HEPES, pH 7.5, 10 nM [^{3}H]N-methyl scopolamine (85 Ci/mmol, Du Pont) with or without 10 μM N-methyl atropine. After 40 min at 37°C, proteins were precipitated with polyethylene glycol and label was measure by liquid scintillation counting.

Thrombin receptors were assayed in 500 μl incubations containing: 10 mM $MgCl_2$, 150 mM NaCl, 0.25% (w/v) BSA, 0.05% (v/v) bacitracin, 0.1 mM AEBSF, 2 μg/ml aprotinin, 7% (v/v) glycerol, 2 mg/ml phosphatidylserine, in Tris HCl (50 mM pH 7.4). [^{125}I]TRP 10 mM (approx. 60,000 cpm/assay) with or without 300 nM unlabelled TRP to define non-specific binding and were carried out for 60 min at 4°C.

2.2.10 Modulation of agonist affinity at M_3 receptor by GTP analogues

1321N1 cells, control or preincubated with 20 μM carbachol for 10 min at 37°C with or without 100 μM BFA were extensively washed before being homogenised and a membrane fraction prepared as described above. The effects of GTP analogues on the IC_{50} values from carbachol displacement curves of specific [^{3}H]NMS binding were assessed in 500 μl assays containing: 10 mM $MgCl_2$, 100 mM NaCl, 20 mM HEPES, pH 7.5, 1 nM [^{3}H]NMS, with or without carbachol (10-3000 μM), GTPγS (100 μM), $GPPCH_2P$ (200 μM), F^- (10mM NaF in the presence of 30 μM $BeCl_2$) or N-

methyl atropine (3 μM). After 60 min at 37 °C, assays were quenched with 750 μl ice-cold 10 mM $MgCl_2$, 100 mM NaCl, 20 mM HEPES pH 7.5, centrifuged and pellets taken for liquid scintillation counting.

2.2.11 Solubilization and immunoprecipitation of wild-type and Asn318 mutant GnRH receptors labelled with [^{125}I]buserelin

Iodination of Buserelin

Ligand binding assays to the GnRH receptor were carried out with the protected GnRH analogue buserelin (D-Ser(Bu^t)^6 des Gly^{10} GnRH-ethylamide, Calbiochem-Novabiochem (U.K.) Ltd., Nottingham) Buserelin was radiolabelled as described for the thrombin ligand TRP. Specific activities of 1100 Ci/mmol were routinely obtained.

COS 7 cells expressing either the wild type or Asn318 mutant GnRH receptor, were preincubated with or without 100 nM GnRH for 15 min. Membranes were prepared from these cells as described above for the 1321N1 cells. The GnRH receptors were solubilized from the crude membrane fraction over a 30 min incubation at 4°C, using ice-cold buffer containing 5 mM CHAPS, 1.5 mM NaCl, AEBSF 1 mM, aprotinin 2 μg/ml, leupeptin 4 μg/ml, pepstatin A 2 μg/ml, soybean trypsin inhibitor 50 μg/ml, NaF 1 mM and sodium orthovanadate 1 mM whilst rolling for 30 min at 4°C. The extracts were adjusted to 0.5M NaCl and pre-cleared of proteins that bind non-specifically to protein G Sepharose as described above. Pre-cleared extracts were incubated with the immunoprecipitating antibodies for 18 hours on a tube roller, at 4°C; anti-ARF1/3 immunoglobulins (10 μl/ml), anti-RhoA IgG (1 μg/ml), or with control antibodies at 1 μg/ml; mouse monoclonal anti-ERK1/2 IgG (Zymed Laboratories) or mouse monoclonal anti-Ras IgG (Transduction

Laboratories), each being reported to recognise the native conformation of their target protein. Antibody-blocking peptides were used in controls at 2 μg/ml. Antibody-antigen complexes were collected as described previously incubation with protein G-Sepharose suspension, and washed the solubilization buffer containing 5 mM CHAPS, 0.5M NaCl and protease/phosphatase inhibitors. Receptors remaining in solution were concentrated by polyethylene glycol precipitation and specific [^{125}I]buserelin binding was assayed in a volume of 500 μl containing 10-60 μg protein, 25 mM Tris HCl pH 7.4, 0.1% (w/v) BSA, 25 pM [^{125}I]buserelin (50,000 cpm) with or without 3 μM GnRH. After 90 min at 4°C, proteins were precipitated with polyethylene glycol and bound ligand was measured by γ-spectrometry.

2.2.12 Modulation of agonist affinity at the wild-type and Asn318 mutant GnRH receptors by GTP analogues

COS 7 cells transfected with wild type or Asn318 Mutant GnRH receptor were seeded into fibronectin-coated tissue culture flasks (5 μg/cm^2). Experiments were carried out by the sequential addition of washes to cells that had been previously equilibrated with [^{125}I]buserelin over 90 min at 4°C in Medium 199 with 0.2% BSA (w/v). Non-specific binding was defined with 3 μM GnRH. The cells were permeabilized with medium 199 containing 22 μM digitonin, and GTP analogues as required, 100μM GTPγS, 200 μM $GPPCH_2P$ or 10 mM NaF in the presence of 30 μM $BeCl_2$. Addition of medium at 37°C caused a rapid initial loss of over 80% of the specifically bound ligand with a $t_{1/2}$ which was indistinguishable between wild-type and Asn318 receptors ($t_{1/2}$ of 1.87 ± 0.11 min, mean ± SEM from 6 typical examples of each receptor type). This corresponds to the marked reduction in GnRH agonist equilibrium binding to membranes resulting

from transfer from 0°C to 37°C (Perrin, M. H., et al. 1989). After 15 min, ligand dissociation from permeabilized cells had reached a slower steady state; a plot of $^{B}/_{Bo}$ against time (15-50 min) then revealed a good fit to a linear component in each case: $t_{1/2}$ control wild-type receptor 22 ± 3 min and control Asn318 receptor 26 ± 3 min (means ± SEM, n = 6).

2.2.13 Ligand-binding studies on the VIP and PACAP receptors

Iodination of Helodermin

Helodermin (Calbiochem-Novabiochem (U.K.) Ltd., Nottingham) was radiolabelled as described above for TRP. Specific activities of 200 μCi/μg were routinely obtained.

Homologous displacement of Helodermin/PACAP-27 from whole cells at 37°C.

CHO cells stably transfected with either the VIP_1, VIP_2, $PACAP_{short}$ or $PACAP_{long}$ receptor cDNAs, were seeded into twelve well plates. The cells were washed twice with warm (37°C) Medium 199 containing 0.2% BSA, and the protease inhibitor, bacitracin (30μg/ml). The 0.5 ml wash was replaced with the assay medium; 0.4 ml of Medium 199/BSA/bacitracin, 50 μl of increasing concentrations of unlabelled ("cold") helodermin/PACAP-27 peptide, and 50 μl of radiolabelled helodermin/PACAP-27 (NEN DuPont, U.K.). The specific activity of [^{125}I]PACAP-27 and [^{125}I]helodermin was 2200 Ci/mmol and 77 Ci/mmol respectively. The iodinated ligands were diluted to 50,000 cpm/50 μl in Med 199/BSA/bacitracin plus the protease inhibitor aprotinin to a final concentration of 10 μg/ml and the addition of the labelled peptide marked the start of the assay. The plates were incubated at 37°C for 10

mins in a shaking water bath. The plates were placed on ice for 10 minutes then the assay medium was immediately removed by aspiration, and the cells were washed three times with ice-cold Earle's Balanced Salt Solution (EBSS) containing 0.1% BSA. Once the third wash was removed, cells were washed with an ice-cold solution of 0.2M glacial acetic acid/0.5M NaCl. The acid strip wash was removed from the wells after 5 minutes and pipetted into eppendorf microtubes. This acid strip procedure has been utilised by a number of groups such as those investigating the gastrin-releasing peptide receptor (Grady, E. F., et al. 1995, Slice, L. W., et al. 1994), and is taken to represent the readily-dissociated externally bound ligand. The cells were then lyzed using 4M NaOH. The lysis solution was left on overnight and then the cells were homogenised and transferred to eppendorf microtubes; this fraction contained the internalised ligand. Gamma radiation was measured by γ-spectrometry. In each plate, 2 wells were washed with warmed EBSS and then the cells were homogenised in a solution of 1% sodium lauryl sulphate for protein determination

2.2.14 Western Blotting

Extracts from 1321N1 cells or male rat anterior hemipituitaries (pre-incubated with 10 µM AT II for 10 min at 37°C) were homogenised and solubilized in the buffer/detergent systems described above for 1321N1 cells. However in the solubilization step, no glycerol or PtdCho was added. Immunoprecipitation was carried out as described above, using sheep anti-ARF1/3 immunoglobulins at 20 µl/ml (peptide at 8 µg/ml), rabbit anti-RhoA IgG at 5 µg/ml (peptide at 20 µg/ml), rabbit anti-M_3 receptor serum globulins (Wall, S. J., et al. 1991), or non-immune rabbit IgG at 2,5 µg/ml and polyclonal anti-AT_1 receptor IgG at 5 µg/ml (Santa

Cruz). Primary antibody complexes were collected with 15-20 µl protein G-Sepharose and washed once with buffer A. Samples were then solubilized by heating the immunoprecipitates to 100°C for 5 min, in 50 µl PAGE lysis buffer containing Tris HCl pH 7.6, 2% sodium dodecyl sulphate and 0.5% β-mercaptoethanol. Samples were flash-frozen to -70°C until ready for electrophoresis and immunoblotting. On the day of electrophoresis the thawed samples were heated for 5 minutes under hot water tap (approx. 50°C) and centrifuged for 2 minutes at room temperature in Eppendorf centrifuge to remove particulate material.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The 7.5% homogeneous polyacrylamide gel pre-cast onto a rigid polyester film (PhastGel Homogeneous 7.5, Pharmacia) was removed from its wrapping and carefully placed onto 80 µl of UHP H_2O on the gel bed of the PhastSystem Separation and Control unit (Pharmacia Biotech AB, Uppsala, Sweden) in such a manner as to exclude air bubbles. The gels have a 13 mm stacking zone and 32 mm separation zone (dimensions; 43 x 50 x 0.45 mm). PhastGel SDS buffer strips were then positioned in the buffer strip holder. The samples were loaded (4 µl) onto the sample strip holder in a humidified chamber and the holder was positioned in the apparatus. Low molecular mass standards of 97, 67, 43, 30, 20.1 and 14.4 kDa (Pharmacia) were used in all separations. The separation program was run until 60 Vh had accumulated (approx. 30 min).

Electroblotting

Immobilon transfer membranes (polyvinyldifluoride, 10 cm x 10 cm, 0.45 µM pore size; Millipore) were cut to size (5 cm x 5 cm) and marked for identification and orientation. The filters were immersed in methanol

and then rinsed in UHP H_2O briefly 5 times. The filters were then shaken for 2 x 5 minutes in UHP H_2O and in blot buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 3 x 5 minutes using the rotary shaker.

Once the proteins had been separated, the separation zone of the gel was cut from its plastic backing using cheesewire. The Immobilon membrane was placed over the separation zone of the gel and the gel was removed from its backing whilst remaining in contact with the Immobilon. Three pieces of PhastTransfer filter paper (50 mm x 50 mm) were soaked in blot buffer and placed on the anode of the PhastSystem Development Unit. More blot buffer was applied to the filters and the Immobilon placed on top of them. Another three filters were then soaked and placed on top of the immobilon/gel. The cathode was then placed on top of the filters and pressed into place gently. The transfer of proteins was carried out using 90 Vh (approx. 45 min).

Immunostaining

The blot was given 5 quick rinses in UHP H_2O before being given 4 x 4 minute washes on the shaker. The blot was then stained for 5 minutes in 0.1% Coomassie blue-R250, 50% methanol before being given two or more 1 minute washes in destain (50% methanol, 10% acetic acid) until the protein bands were clearly visible against a clear or pale background. Several 1 minute washes in UHP H_2O were then carried out to stop the destaining process and remove residual methanol. The blot was then marked and the lanes cut up as appropriate. The blot was washed two or more times for 2 minutes in methanol until the bands were no longer visible. A further 3 x 4 minute washes in methanol were carried out

before the blot was given 5 x 5 minute washes in UHP H_2O to remove residual methanol.

The blot was incubated overnight at 4°C in either 5% BSA (w/v)/PBS or 5% Marvel. The blot was given 2 quick rinses and 5 x 5 minute washes in PBS/0.1% polyoxyethylenesorbitan monolaurate (Tween-20, Sigma). The blot was incubated overnight with the primary antibody in 0.25% BSA/PBS/0.05% Tween-20. An appropriate blocking peptide was mixed with the primary antibody for use as a control.

The blot was given two quick rinses and five x 5 minute washes in 0.25% BSA/PBS/0.1% Tween-20 and incubated for 1 hour with horseradish peroxidase (HRP)-conjugated sheep-derived anti-mouse or donkey-derived anti-rabbit polyclonal IgG antiserum (Scottish Antibody Production Unit). Secondary antibodies were diluted 1:5000 or 1:2000 for anti-mouse and anti-rabbit polyclonal IgG respectively. Dilution was in 0.25% BSA/PBS/0.05% Tween-20 and then the blot was then rinsed as previously.

In the darkroom the blot was incubated for 1 minute in 2.5 ml of a 1:1 mixture of Enhanced Chemi-Luminescence (ECL) solutions A and B (Amersham). Excess liquid was removed from the blot by touching the edge with a mediwipe tissue. The blot was then sealed in clingfilm and exposed against ECL film for between 30 seconds and 5 minutes. The film was developed by incubation for 4 minutes in Phenisol developer, rinsing in water, and a 1 minute incubation in Hypam fixer followed by a minimum of 2 minutes in running water.

2.2.15 Assay of protein concentration

The concentration of protein in wells was determined using the Pierce Bicinchoninic acid (BCA) system (Pierce). A 10 µl sample taken from homogenised tissue was spotted into a 96 well microplate (Greiner) and 300 µl of BCA protein assay reagent was added, the plate was warmed to 37°C for 30 min. The absorbance of the sample was measured at 590 nm using a Microplate Biokinetics Reader EL 312e (BIO TEK Instruments, Vermont, USA). Sample values were compared to a standard curve constructed using an albumin standard.

2.2.16 Data analysis

All values are expressed as mean ± standard error of the mean (SEM) from data obtained from a number (n) of individual experiments. Statistical analyses were carried out using the Mann-Whitney U-test unless otherwise stated. The concentration of drug which could produce 50% of the maximal response (EC_{50} value) and the concentration of inhibitors which inhibit 50% of a stimulus-evoked response (IC_{50}) were assessed by fitting the data with a non-linear, error weighted, iterative curve-fitting programme, P.fit (Biosoft, Cambridge, U.K.) and represented as mean ± SEM.

CHAPTER 3

Activation of PLD by rhodopsin family GPCRs native to 1321N1 cells and anterior pituitary tissue

3.1 INTRODUCTION

The majority of transmembrane signal transduction in response to hormones and neurotransmitters is mediated by G protein-coupled receptors (GPCRs). Moreover, GPCRs are the principal signal transducers of sight and smell. GPCRs are integral membrane proteins characterised by seven hydrophobic stretches of amino acids that are predicted to form transmembrane domain (TMD) α helices, connected by alternate extra and intracellular loops (Baldwin, J. M. 1993, Baldwin, J. M. 1994, Dohlman, H. G., et al. 1991). Based on certain key sequences, GPCRs can be divided into three major subfamilies, receptors related to rhodopsin (class I), receptors related to those for VIP/calcitonin (class II) and the metabotropic glutamate $GABA_B$ and Ca^{2+} sensor receptors (class III). The rhodopsin subfamily is the most extensively studied and the position of the seven transmembrane helices (as proposed by Baldwin, and based upon the low resolution projection map for rhodopsin produced by Schertler (Baldwin, J. M. 1993, Baldwin, J. M. 1994, Schertler, G. F. X., et al. 1993)), points to the anticlockwise arrangement of the helices and the close proximity of TM helices I, II and VII, as viewed from the extracellular surface (Baldwin, J. M. 1993, Baldwin, J. M. 1994, Schertler, G. F. X., et al. 1993). Although these receptors all possess the basic seven TMD structure, the number of highly conserved amino acids shared by the rhodopsin subfamily of GPCRs is relatively few (Probst, W. C., et al. 1992, Savarese, T. M. and C. M. Fraser 1992) and the conservation of any amino acids points to an important role in receptor function. One such conserved motif is the characteristic $N/DPX_{2-3}Y$ sequence that is completely conserved in the rhodopsin family, with $NPX_{2-3}Y$ present in TMD 7 of 95% of class I GPCRs, the remaining 5% of receptors contain an aspartate residue at the

first position (Probst, W. C., et al. 1992). Models of aminergic GPCRs suggest that the $NPX_{2-3}Y$ sequence is ideally placed to mediate any signal from the agonist-induced conformational changes in the ligand binding region (Donnelly, D., et al. 1994, Findlay, J. B. C., et al. 1993). The $NPX_{2-3}Y$ motif is similar to the NPXY internalisation sequence that was first described in the cytoplasmic segment of the low-density lipoprotein receptor (Trowbridge, I. S., et al. 1993). In recent studies, mutagenesis pointed to a role for the tyrosine of the motif in regulation of agonist-induced internalisation of the β_2 adrenergic receptor (Barak, L. S., et al. 1994). However further studies utilising mutagenesis of the β_2-adrenergic (β_2), 5-HT_{2A}, angiotensin II type 1 (AT_1), neurokinin-1 (NK_1) and thyrotropin-releasing hormone (TRH) receptors demonstrated that the $NPX_{2-3}Y$ motif is not an generic internalisation motif, but it appears to maintain the structural integrity of the receptor, allowing the receptor to interact properly with both agonist and G proteins and thus transduce extracellular signals across the membrane (Barak, L. S., et al. 1995, Barak, L. S., et al. 1994, Böhm, S. K., et al. 1997, Hunyady, L., et al. 1995, Laporte, S. A., et al. 1996, Perlman, J. H., et al. 1997, Sealfon, S. C., et al. 1995). Indeed as demonstrated in the reports of receptor function, mutation of the conserved tyrosine in the β_2, NK_1 and AT_1 receptors to alanine significantly inhibited the respective stimulation of adenylate cyclase, calcium (Ca^{2+}) mobilisation and inositol phosphate (InsP) production (Barak, L. S., et al. 1995, Böhm, S. K., et al. 1997, Laporte, S. A., et al. 1996). Moreover the replacement of the asparagine residue in the β_2, 5-HT_{2A}, AT_1, and TRH receptors with alanine abolished the adenylate cyclase activity of the β_2 receptor and either abolished or significantly reduced the InsP responses of the remaining receptors (Barak, L. S., et al. 1995, Hunyady, L., et al. 1995, Perlman, J. H., et al. 1997, Sealfon, S. C., et al.

1995). Receptors which contain the alternative $DPX_{2-3}Y$ motif have not been the focus of such a detailed analysis of the role that this conserved amino acid sequence may have in receptor conformation and signal transduction capabilities. However, studies on the gonadotropin-releasing hormone (GnRH) receptor demonstrate that site-directed mutagenesis of the aspartate at position 318, to asparagine (which is conserved in 95% of the rhodopsin receptor family) attenuated coupling of the receptor to the phospholipase C/InsP breakdown pathway (Awara, W. M., et al. 1996, Mitchell, R., et al. 1998, Sealfon, S. C. and R. P. Millar 1995, Zhou, W. C., et al. 1994). In further studies (Chapter 4) on the receptor motifs determining receptor linkages to PLD activation, the GnRH receptor was used as a prototypical $DPX_{2-3}Y$-containing receptor, and the wild type (wt) receptor's ability to transduce signals in response to agonist was compared to a mutant restoring the asparagine at position 318. The wt and Asn318 mutant GnRH receptors, expressed in COS-7 cells were compared to the wt5-HT_{2A} ($NPX_{2-3}Y$) and mutant ($DPX_{2-3}Y$) receptor, carrying the reciprocal mutation. Modelling of GPCRs and other proline containing proteins, suggest that as a result of ligand binding the $N/DPX_{2-3}Y$ motif can act as a flexible hinge, around which the TMD 7 can move (Konvicka, K., et al. 1998, Luo, X., et al. 1994, Trumpp-Kallmeyer, S., et al. 1992, Wess, J., et al. 1993), possibly involving cis-trans isomerization of the Asn-Pro peptide bond (Brandl, C. J. and C. M. Deber 1986, Wess, J., et al. 1993, Williams, K. A. and C. M. Deber 1991). The interaction of TMD 7 with the other TMDs is thought to lead to torsion in the α-helical stretches of TMD 7 and consequently the exposure of the regions of the receptor that couple to G proteins (Luo, X., et al. 1994). The region of the receptor around TMDs 5 and 6, and the adjoining third intracellular loop, are expected to be the focus of this movement, which is a domain well

known to be crucial in receptor-G protein coupling (Dohlman, H. G., et al. 1991, Gudermann, T., et al. 1996, Savarese, T. M., et al. 1992, Strader, C. D., et al. 1994, Wess, J. 1997).

The activation of PLD by native $NPX_{2-3}Y$ and $DPX_{2-3}Y$ receptors was studied in the 1321N1 cell line derived from primary cultures of a cerebral glioma multiforme (Foster, S. J. and J. P. Perkins 1977, Macintyre, E. H., et al. 1972). The 1321N1 cell line expresses $NPX_{2-3}Y$ receptors, such as the M_3 muscarinic, H_1 histamine and B_2 bradykinin receptors (Hepler, J. R., et al. 1987, Masters, S. B., et al. 1984, Nakahata, N., et al. 1985, Wall, S. J., et al. 1991) and the $DPX_{2-3}Y$-containing thromboxane A_2 and thrombin receptors (Nakahata, N., et al. 1989, Nieto, M., et al. 1994). All of these receptors couple tightly to G proteins of the $G_{q/11}$ family, and display the expected stimulation of phosphoinositide breakdown in these cells. In addition the activation of PLD by both $NPX_{2-3}Y$ and $DPX_{2-3}Y$ receptors in 1321N1 cells has been reported (Nieto, M., et al. 1994). The involvement of small G proteins of the ARF and Rho families in the activation of PLD was described recently, (Bowman, E. P., et al. 1993, Brown, H. A., et al. 1993, Cockcroft, S., et al. 1994), and as briefly outlined above, the $N/DPX_{2-3}Y$ sequence is proposed as a regulator of receptor conformational change and G protein activation. The direct and/or indirect coupling of ARF and Rho proteins to the $N/DPX_{2-3}Y$ containing receptors, and activation of PLD was the focus of this study. The results demonstrate that small differences in the TMD 7 architecture can have dramatic effects on the receptor signal transduction characteristics, and the findings presented here are consistent with an important role for the $N/DPX_{2-3}Y$ motif in regulation of phosphatidylcholine breakdown by PLD.

3.2 RESULTS

Separation of [^{3}H]PtdBut by thin layer chromatography

The production of [^{3}H]PtdBut by the activation of PLD was measured as described in Chapter 2. Figure 3.1 shows the running position of [^{3}H]PtdBut in LK5D thin layer chromatography plates, in a typical experiment. The results show the increase in [^{3}H]PtdBut production in 1321N1 cells stimulated with 200 μM carbachol or 0.5 units/ml thrombin, and compared with basal activity in these cells. The increase in agonist-evoked [^{3}H]PtdBut production is shown for duplicate treatments of 12-well plates containing 1321N1 cells.

Receptor-mediated activation of PLD: studies with selective signal transduction inhibitors

The activation of PLD by selected receptors, endogenously expressed by the 1321N1 human astrocytoma cell line, was challenged with brefeldin A (BFA), a fungal metabolite which acts as a selective inhibitor of guanine nucleotide exchange on ARF (Donaldson, J., et al. 1992, Helms, J. B. and J. E. Rothman 1992). These responses fall into 2 clear categories as described recently by Mitchell *et al.* (Mitchell, R., et al. 1998). The M_3 muscarinic, H_1 histamine, B_2 bradykinin, thrombin and thromboxane A_2 (TXA_2) receptors (Debacker, M. D., et al. 1993, Hess, J. F., et al. 1992, Hirata, M., et al. 1991, Peralta, E. G., et al. 1987, Vu, T. K. H., et al. 1991) all activate PLD potently after exposure to the appropriate agonist: carbachol, histamine, bradykinin, thrombin and U46619 (11α,9α-epoxymethano-PGH_2) respectively. We showed that the bradykinin receptor in these cells is most likely to be of the B_2 subtype, as the B_1 receptor-selective agonist kallidin, did not elicit any increase in [^{3}H]PtdBut production over basal

activity. PLD activity, as measured by the increase in [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut), stimulated by the M_3 muscarinic, H_1 histamine and B_2 bradykinin receptors was sensitive to BFA, suggesting an involvement of ARF. However the thrombin and thromboxane A_2 receptor-mediated activation of PLD , measured under identical conditions, was BFA-resistant (Figure 3.2). The PLD responses elicited by the M_3, B_2 and H_1 receptors were all significantly inhibited by 50 μM BFA ($p<0.05$), and in the presence of 200 μM BFA the PLD activity was reduced to 15 ± 12%, 27 ± 7% and 32 ± 8%, of the maximal stimulated responses respectively. The IC_{50} values for BFA (concentration of inhibitor and/or antagonist required to block 50% of maximum response) on the M_3, B_2 and H_1 receptors were 72 ± 11, 78 ± 15 and 82 ± 13 μM respectively, whereas the thrombin and thromboxane A2 receptors were resistant to BFA up to 200 μM, with 79 ± 10% and 89 ± 7% of maximal responses remaining at that concentration.

The M_3 and thrombin receptors were further studied as prototypical examples of $NPX_{2-3}Y$ and $DPX_{2-3}Y$ motif-containing receptors. The concurrent activation of phospholipases C (PLC) and D upon agonist stimulation of PLC-coupled receptors has been reviewed by Bocckino and Exton (Bocckino, S. B. and J. H. Exton 1996). Moreover, the dependence of PLD on PLC activation downstream of receptor for EGF, PDGF and GnRH further implicates a role for PLC in those examples of receptor-evoked phosphatidylcholine metabolism (Yeo, E.-J. and J. H. Exton 1995, Yeo, E.-J., et al. 1994, Zheng, L., et al. 1994). The results presented in Figure 3.3 suggest that the PLD response downstream of the thrombin, but not M_3 receptor is dependent on the activation of phospholipase C (PLC) as there was significant attenuation of the thrombin-induced PLD response, by the

selective PLC inhibitor U73122 (Bleasdale, J. E., et al. 1989, Bleasdale, J. E., et al. 1990). In the presence of the highest concentration of U73122 used (20 μM), there was 66 ± 8% inhibition of the thrombin-induced PLD activity with an IC_{50} for U73122 of 14 ± 4 μM. In contrast, 20 μM U73122 caused only a 20 ± 13% inhibition of the M_3 receptor-stimulated response ($p<0.05$, n = 4-10).

We could find no evidence for involvement of the $G_{i/o}$ proteins in the activation of PLD by either the M_3 or thrombin receptors. Pre-treatment of 1321N1 cells with pertussis toxin (PTx, 200 ng/ml, 18h) did not reduce M_3 or thrombin receptor-stimulated PLD activity (Figure 3.4). Ptx inactivates $G_{i/o}$ proteins by ADP-ribosylation of the cysteine residue, four amino acids from the carboxyl terminus of the α-subunit (Neer, E. J., et al. 1984, Tsai, S. C., et al. 1984, West Jr, R. E., et al. 1985). The involvement of Rho family G proteins in the activation of PLD by the M_3 and thrombin receptors was investigated using C3 ADP-ribosyltransferase (C3 exoenzyme from Clostridium *botulinum*)(Aktories, K., et al. 1988, Aktories, K., et al. 1987). This enzyme ADP-ribosylates RhoA, B and C on asparagine-41 and inactivates them (Paterson, H. F., et al. 1990, Ridley, A. J. and A. Hall 1992, Sekine, A., et al. 1989). A role for Rho in the activation of PLD was reported by Schmidt *et al.*, who stably expressed M_3 receptors in Human Embryonic Kidney (HEK) cells (Schmidt, M., et al. 1996). Also the activation of Rho by the thrombin receptor in 1321N1 cells has recently been reported, where thrombin-stimulated cell rounding and increase in DNA synthesis is blocked by pretreatment of cells with C3 exoenzyme (Majumdar, M., et al. 1998). In addition to the C3 exoenzyme studies, a wild type (wt) and functionally negative construct (CMV5Asn19) of RhoA, were transfected into 1321N1 cells, as molecular

tools to enhance or attenuate of Rho function (Zhang, S., et al. 1995). C3 exoenzyme and the CMV5Asn19 RhoA construct significantly reduced the M_3 but not the thrombin receptor-mediated activation of PLD (Figure 3.4). C3 exoenzyme pretreament produced a 60 ± 15% inhibition of carbachol-induced PLD activation, while the CMV5Asn19 RhoA construct reduced the PLD response to 64 ± 11% of maximum. The potency of the negative Rho construct depends on the efficient translation of enough protein to compete with the endogenous Rho in the activation of PLD, the lower level of inhibition produced by CMV5Asn19 RhoA may reflect a low level of functionally active protein present within the cell. The thrombin-induced PLD responses in the presence of C3 exoenzyme and CMV5Asn19 RhoA were 87 ± 13% and 122 ± 21% of maximum respectively. As shown in Figure 3.4, the ability of wtRhoA to augment the activation of PLD by low concentrations of carbachol but not thrombin, is consistent with a role for Rho in M_3 and not thrombin receptor-mediated PLD activation. Pre-treatment of 1321N1 cells with wtRhoA increased the PLD response to 20 μM carbachol to 169 ± 12% of that with carbachol alone. However the PLD response to similar wtRhoA pre-treatment and 0.2 Units/ml thrombin as agonist, was only 104 ± 8% of the PLD activity in the presence of thrombin alone. Therefore it appears that two groups of $G_{q/11}$-linked receptors exist in 1321N1 cells; those whose PLD responses were either sensitive or resistant to BFA, C3 exoenzyme and the negative CMV5Asn19 RhoA construct. The receptors which couple to PLD in a manner sensitive to the small G protein inhibitors (M_3, H_1 and B_2) all contain the $NPX_{2-3}Y$ motif in TMD 7, while the resistant group of receptors, contain the alternative $DPX_{2-3}Y$ motif (thrombin, TXA_2).

Receptor association with small G proteins: ligand binding studies on ARF/RhoA immunoprecipitates

The different pharmacology of PLD activation displayed by the M_3 and thrombin receptors suggests a divergence in their ability to couple with ARF and RhoA. The possibility that the M_3 and/or thrombin receptors form close associations with small G proteins in their activation of PLD was tested by attempting the co-immunoprecipitation of ARF1/3 and/or RhoA together with any associated receptor binding sites. The results in Figure 3.5 show that M_3 receptors, as denoted by specific [^{3}H]N-methyl scopolamine ([^{3}H]NMS) binding, could be solubilized from the membrane and immunoprecipitated using sheep ARF1/3 or rabbit Rho polyclonal antisera (Laudanna, C., et al. 1996, Martin, A., et al. 1996). The co-immunoprecipitation of specific [^{3}H]NMS binding was dependent upon prior cell exposure to carbachol (100 μM, 10 min). This 'priming' with agonist is proposed to cause immediate events within the cell which include allowing the close association of the receptor with small G proteins e.g. translocation of monomeric G proteins to the nucleus. Priming causes a significant 4.8 ± 0.7 fold increase in specific ARF 1/3-precipitated [^{3}H]NMS binding ($p<0.05$, n = 6-9), compared to the unprimed control, and this rise was inhibited by 84 ± 5% in the presence of excess ARF 1/3 blocking peptide. Moreover the pre-treatment of cells with 100 μM BFA during priming also reduced by 74 ± 19% the level of [^{3}H]NMS binding co-precipitated using ARF 1/3 antiserum. Incubation with polyclonal Rho antibodies significantly co-precipitated specific [^{3}H]NMS binding sites from the solubilized extract of primed 1321N1 cells, as shown in Figure 3.5. Exposure of cells to carbachol caused an 1.8 ± 0.3 fold increase in [^{3}H]NMS binding associated with Rho immunoprecipitates,

and this was specifically reduced, using excess blocking peptide, by 57 ± 12%. A small level of [^{3}H]NMS binding was associated with control non-immune sheep (NIS) and non-immune rabbit (NIR) IgG antibodies, used singly or in combination, and this did not rise in response to priming with carbachol. The number of [^{3}H]NMS binding sites co-immunoprecipitated using ARF 1/3 and Rho antibodies either alone or in combination, was significantly higher than using a combination of non-immune reagents (NIR + NIS), but less than additive. Together ARF + NIR and Rho + NIS co-immunoprecipitated 0.55 ± 0.09% and 0.42 ± 0.08% of the added [^{3}H]NMS ligand respectively. In combination, ARF + Rho immunoprecipitates retained specific binding of 0.75 ± 0.14% of the added [^{3}H]NMS ligand (Figure 3.5). As summarised in Figure 3.6, binding of the specific thrombin ligand [^{125}I]Ala-pFPhe-Arg-Cha-HArg-Tyr-NH_2 ([^{125}I]TRP) (Feng, D. M., et al. 1995), was not detectable in ARF 1/3 or Rho immunoprecipitates from 1321N1 cells primed with the thrombin agonist Ser-Phe-Leu-Arg-Asn-NH_2. Although high affinity specific binding of the ligand could be detected in 1321N1 membrane preparation and solubilized extract (R. Mitchell, unpublished observations).

Immunodetection of receptor association with ARF and Rho in 1321N1 cells

The activation of PLD by the M_3 receptor, in an ARF- and RhoA-dependent manner, and the presence of [^{3}H]NMS binding sites in immunoprecipitates produced using carbachol-primed 1321N1 cells and ARF and RhoA antibodies is indicative of a close association, if not direct interaction of the M_3 receptor and small G proteins. Immunoblotting was used to further investigate the direct association of receptor and G proteins and how this is modulated by exposure to agonist.

Immunoprecipitates of ARF1/3, and RhoA from 1321N1 cells, prepared using polyclonal antisera, were probed with monoclonal antibodies to ARF and Rho to authenticate the specific immunoprecipitation of the correct proteins. The results presented in Figures 3.7a and b, show that the sheep polyclonal anti-ARF1/3 and rabbit polyclonal anti-RhoA antibodies, correctly immunoprecipitated the appropriate proteins, and that this was dependent on exposure to agonist and blocked by the presence of excess peptide antigen. The co-precipitation of ARF and Rho, using a rabbit polyclonal antiserum directed against the M_3 receptor (Wall, S. J., et al. 1991), provides further proof for the direct association of the receptor with small G proteins and is consistent with the presence of [^{3}H]NMS binding sites in ARF1/3 and RhoA immunoprecipitates (Figure 3.8a and b). The presence of ARF and Rho in the M_3 receptor immunoprecipitates was also dependent on priming with carbachol and a non-immune rabbit IgG control did not co-precipitate any detectable immunoreactive ARF or Rho.

Studies on $NPX_{2\text{-}3}Y$ and $DPX_{2\text{-}3}Y$ receptors in normal tissue from rat anterior pituitary gland

In order to substantiate that similar coupling events occur in normal physiological tissue as opposed to a clonal cell line, experiments were carried out on the AT_{1A} ($NPX_{2\text{-}3}Y$) receptor and the GnRH ($DPX_{2\text{-}3}Y$) receptor in male rat anterior pituitary tissue. Angiotensin II-stimulated PLD activity in rat anterior pituitary slices was BFA-sensitive, with an IC_{50} of 58 ± 5 μM, which is comparable with that of the M_3 receptor whereas (as predicted) the response to GnRH was unaffected up to 200 μM (see Figure 3.9). Polyclonal antibodies raised against the angiotensin II type 1 (AT_1) receptor were used to isolate the receptor and the

immunoprecipitates from solubilized extracts of angiotensin II-primed rat anterior pituitaries (which express AT_1 receptors exclusively, (Bottari, S. P., et al. 1993)), were probed for the presence of ARF. The receptor closely associated with ARF in an agonist-dependent manner as shown in Figure 3.10, and this was clearly blocked by the presence of excess peptide antigen during the immunoprecipitation procedure.

Receptor-mediated activation of PLD and agonist recognition: effect of GTP analogues

The specific association of the M_3 receptor with ARF and Rho during the stimulation of receptor with carbachol as shown above, was further investigated by using GTP analogues to activate G proteins and modulate the receptor affinity for ligand. This work summarises experiments done with Rory Mitchell (R. Mitchell and D. McCulloch: manuscript in preparation)

In membranes from 1321N1 cells (with or without prior priming of the cells with 100 μM carbachol for 10 mins at 37°C), the concentration of carbachol required to displace bound [^{3}H]NMS was monitored, in the presence of non-hydrolyzable GTP analogues. The results presented in Table 3.1a show that treatment of 1321N1 cells with; guanosine 5'-O-(3-thio)-triphosphate (GTPγS), beryllium fluoride (BeF_3^-, an isostere of aluminium fluoride, (AlF_4^-), both of which mimic GTP) or guanosine 5'-[βγ–methylene]triphosphate ($GPPCH_2P$), all decreased significantly the M_3 receptor's affinity for carbachol. The effect of incubation with GTPγS, BeF_3^- and $GPPCH_2P$, at the concentration used, was an increase in the IC_{50} for carbachol to displace [^{3}H]NMS binding by 5.23 ± 0.97, 4.06 ± 0.80 and 2.31 ± 0.34 fold, compared to the IC_{50} determined in the absence of any

GTP analogue. Prior exposure to 100 μM carbachol however had different consequences, on the inferred ability of the three GTP analogues to activate cellular G proteins and produce a receptor state with low affinity for agonist. Priming did not significantly change the increase in carbachol IC_{50} induced by GTPγS, whereas the BeF_3^-effect was significantly diminished and the result of $GPPCH_2P$ stimulation was potentiated, the IC_{50} values are respectively 4.48 ± 0.34, 1.82 ± 0.31 and 3.46 ± 0.40 fold increase in IC_{50} compared to unprimed control. Thus priming of 1321N1 cells with carbachol appears to selectively potentiate the activity of $GPPCH_2P$-sensitive G proteins, with a 1.5 ± 0.17 fold increase in the IC_{50} for carbachol compared to unprimed cells. The concurrent inclusion of 100 μM BFA blocked the potentiating effect of carbachol priming and lowered the fold increase in IC_{50} elicited by $GPPCH_2P$ to 1.97 ± 0.34, a 57 ± 10% decrease in the $GPPCH_2P$-stimulated increase in agonist IC_{50} ($p<0.05$, n = 4, results not shown). Moreover priming did not appear to stimulate the population of G proteins activated by BeF_3^- treatment, and the prior exposure to carbachol partially restored the M_3 receptors to the high affinity state absent in BeF_3^- treated cells. Priming of BeF_3^--stimulated cells produced a 56 ± 17% drop in the IC_{50} for carbachol, this is consistent with priming reducing the receptor linkage to G proteins that are selectively activated by $BeF3^-$. The effect of GTPγS on receptor affinity was not influenced by priming and this is consistent with the idea that both the $GPPCH_2P$- BeF_3^-sensitive G proteins are activated by this analogue.

The activation of PLD by GTPγS, BeF_3^- and $GPPCH_2P$ in digitonin-permeabilized 1321N1 cells could also be modulated by agonist priming, and the differential effects of BFA and C3 exoenzyme observed on these

responses are consistent with a role for ARF and Rho in PLD activation by M_3 receptors. Results presented in Table 3.1b show that in unprimed 1321N1 cells, GTPγS, BeF_3^- and $GPPCH_2P$, at the concentrations used, increase PLD activity 150 ± 9, 115 ± 14, and 48 ± 8% as compared to basal activity. GTPγS and BeF_3^- were 3.10 ± 0.05 and 2.40 ± 0.07 fold more effective than $GPPCH_2P$, at stimulating PLD activity. Pre-treatment of cells with BFA or C3 exoenzyme had no significant effect on the BeF_3^--stimulated PLD activity, whilst significantly reducing the $GPPCH_2P$-induced increase in PLD activity to 31 ± 3 and 27 ± 5% increase over basal control respectively. Therefore the βγ-methylene analogue of GTP does appear to show a selectivity for the small G proteins ARF and Rho, not exhibited by BeF_3^-, as suggested by Gill and Coburn (Gill, D. M. and J. Coburn 1987). The activation of PLD by GTP analogues after agonist priming, as with the experiments on receptor affinity described above, implicates the selective activation of $GPPCH_2P$-sensitive G proteins. The prior exposure to 100 μM carbachol had no marked effect on the GTPγS-stimulated increase in PLD activity, as compared with the unprimed control, whereas priming significantly reduced the BeF_3^--induced PLD activity by 32 ± 9% of the unprimed response, to a 78 ± 10% increase over basal. As with the results from the study of receptor agonist affinity, priming potentiates the $GPPCH_2P$ effect, this time on phosphatidylcholine breakdown, with a 56 ± 7% increase in the $GPPCH_2P$-stimulated PLD response after priming, to a 110 ± 12% increase in PLD activity over basal control. The pre-incubation with BFA and C3 exoenzyme had no effect on the BeF_3^--stimulated response, while both inhibitors significantly attenuated the augmented $GPPCH_2P$-induced PLD activity. Treatment of cells with 100 μM BFA (after priming) reduced the $GPPCH_2P$-stimulated PLD activity by 65 ± 8%, to a 38 ± 9 % increase over basal. The effect of C3

exoenzyme treatment was equally striking with a 79 ± 6% inhibition of the $GPPCH_2P$-induced response to 23 ± 7% increase in PLD activity over basal. The ability of $GPPCH_2P$ to act apparently as a selective activator of PLD through ARF and Rho is increased after exposure to agonist, as might be expected due to the reported agonist-stimulated translocation of ARF1/3 and RhoA from the cytoplasm to cell membranes, where interaction with relevant targets can occur (Cavenagh, M. M., et al. 1996, Houle, M. G., et al. 1995, Malcolm, K. C., et al. 1996, Rümenapp, U., et al. 1995). Meanwhile the exposure to carbachol is predicted to lead to receptor-mediated activation of the trimeric G proteins, followed by their breakdown into α and $\beta\gamma$ subunits and thus a reduction in the ability of BeF_3^- to activate PLD.

3.3 DISCUSSION

Pathways to PLD

The activation of PLD by agonist stimulation of the M_3, B_2, H_1, TXA_2 and thrombin receptors in 1321N1 cells was differentially inhibited by BFA, as shown in Figure 3.2. The endogenous M_3 and thrombin receptors in 1321N1 cells have been shown to activate PLD (Nieto, M., et al. 1994). The PLD responses mediated by activation of the M_3, B_2 and H_1 receptor were significantly reduced by BFA implicating a role for ARF. In contrast the TXA_2 and thrombin receptors activated PLD in a BFA-insensitive and so ARF-independent manner. Work done on the M_3 receptor expressed in human embryonic kidney (HEK) cells also found that the PLD response evoked by the M_3 receptor was BFA-sensitive (Rümenapp, U., et al. 1995). These two groups of receptors differ in one important respect, as described

above, the $NPX_{2-3}Y$ sequence motif within the TMD 7 of the M_3, B_2 and H_1 receptors and the alternative $DPX_{2-3}Y$ motif in the TXA_2 and thrombin receptors (Debacker, M. D., et al. 1993, Hess, J. F., et al. 1992, Hirata, M., et al. 1991, Peralta, E. G., et al. 1987, Probst, W. C., et al. 1992, Vu, T. K. H., et al. 1991). Thus the different conserved sequences in the transmembrane α-helix might affect the receptor's capacity to couple to PLD through ARF, and other small G proteins. The variation in the amino acid composition of TMD 7, does not appear to have a major influence on the coupling of the M_3, B_2, H_1, TXA_2 and thrombin receptors to $G_{q/11}$ and the resulting breakdown of inositol phosphates by PLC, since they all readily activate the pathway (as described by previous studies carried out on the receptors in 1321N1 cells) (Hepler, J. R., et al. 1987, Jones, L. G., et al. 1989, Masters, S. B., et al. 1984, Nakahata, N., et al. 1985, Nakahata, N., et al. 1989, Nieto, M., et al. 1994). The sound coupling of the M_3, B_2, H_1, TXA_2 and thrombin receptors to PLC, and the involvement of PLC in the activation of PLD as described in other systems, such as the PDGF, EGF and GnRH receptors (Yeo, E.-J. and J. H. Exton 1995, Yeo, E.-J., et al. 1994, Zheng, L., et al. 1994), suggested the possibility that PLD activation by the M_3, B_2, H_1, TXA_2 and thrombin receptors might be downstream of phosphoinositide hydrolysis. The M_3 and thrombin receptors were used as prototypical examples of an $NPX_{2-3}Y$ and $DPX_{2-3}Y$-containing receptors. The results of experiments using the selective PLC inhibitor U73122, presented in Figure 3.3 suggest that the thrombin receptor, but not the M_3 receptor, activates PLD primarily through PLC activation. The agonist-stimulated M_3 and thrombin receptors both stimulate PLC and bring about the almost identical redistribution of PKCα and ε, the only PKC isoforms detectable in 1321N1 cells (Nieto, M., et al. 1994, Trilivas, I., et al. 1991). Furthermore the downregulation of PKC isoforms in 1321N1 cells by prolonged

treatment with phorbol 12-myristate 13-acetate (PMA) blocked the carbachol-stimulated activation of PLD (Martinson, E. A., et al. 1989, Martinson, E. A., et al. 1990). This is in agreement with the role of PKC-α as a co-factor for the activation of PLD. This role of PKC is proposed by various investigators to be independent of protein phosphorylation (Conricode, K. M., et al. 1992, Hammond, S. M., et al. 1997, Ohguchi, K., et al. 1995, Ohguchi, K., et al. 1996, Singer, W. D., et al. 1995, Singer, W. D., et al. 1996). Therefore the stimulation of PLD by the thrombin receptor in 1321N1 requires the activation of PLC with a probable rise in PKC activity as reported for the activation of PLD by thrombin in human endothelial and C2C12 myoblast cells (Garcia, J. G. N., et al. 1992, Vasta, V., et al. 1998). However the M_3 receptor appears to activate PLD largely via an alternative, small G protein route with much lesser dependence on phosphoinositide hydrolysis, yet may still require PKC acting as a synergistic cofactor, perhaps independent of its kinase activity. Diglyceride production by M_3 receptor stimulation of PLD and phosphatidic acid phosphohydrolase (PPH) may be enough to cause PKC translocation in the presence of U73122, otherwise any residual PLC activity not blocked by U73122 may cause the translocation. The apparent absence of thrombin receptor coupling to ARF and RhoA in its activation of PLD, may be a reflection of its different alpha-helical structure in TMD 7.

The coupling of the M_3 and thrombin receptors to proteins of the $G_{i/o}$ family has been reported. The selective activation of $G\alpha_{i1}$ and $G\alpha_{i3}$ by the M_1, M_2 and M_3 receptors expressed in HEK cells, and the binding of GTPγS to G proteins stimulated by carbachol binding on transfected M_3 receptors in chinese hamster ovary (CHO) cells, was inhibited by PTx

(Burford, N. T., et al. 1995, Offermanns, S., et al. 1994). Moreover the activation of a PLC-mediated chloride current in *Xenopus* oocytes by the M_3 receptor was enhanced by co-expression of $G\alpha_{o1}$ (Fujii, K., et al. 1996). Thrombin-stimulated activation the p^{21Ras}/MAP kinase pathway via $G_{i/o}$ proteins is well reported. The rapid and transient activation of Ras by the thrombin receptor in CCL39 cells, is sensitive to pertussis toxin (van Corven, E. J., et al. 1993). Furthermore the mitogenic effect of thrombin in 1321N1 cells is dependent on the activation of Ras (LaMorte, V. J., et al. 1993). In addition stimulation of Ca^{2+} release and DNA synthesis in CCL39 cells by a peptide agonist of the thrombin receptor, was inhibited by micro-injection of antibodies to both $G\alpha_q$ and $G\alpha_o$ (Baffy, G., et al. 1994). Thrombin also inhibits agonist-stimulated adenylate cyclase activity in both membrane preparations and intact human platelets (Brass, L. F., et al. 1991), and the activation of PLC by thrombin, in chinese hamster CCL39 cells was sensitive to pretreatment with PTx (Paris, S. and J. Pouyssegur 1986). However the results presented in this study demonstrate that there was no involvement of pertussis-sensitive G proteins from the $G_{i/o}$ family in the activation of PLD by either the M_3 or thrombin receptors. Incubation of 1321N1 cells with 200 ng/ml of PTx had no significant effect on the carbachol and/or thrombin-evoked PLD activity (Figure 3.4). Previous reports have also indicated that the M_3 receptor in 1321N1 cells do not interact with $G_{i/o}$ proteins in the receptor stimulation of phosphoinositide hydrolysis and activation of a cAMP phosphodiesterase (Hughes, A. R., et al. 1984, Masters, S. B., et al. 1985).

The involvement of the small G protein, Rho, in the activation of PLD was first reported by Bowman *et al.*, who demonstrated that the stimulation of PLD by GTPγS in neutrophil cell lysates was inhibited by

the Rho GDP-dissociation inhibitor (GDI) (Bowman, E. P., et al. 1993). This work was confirmed by subsequent studies using membranes from HL-60 cells, neutrophils, rat liver (Kwak, J. Y., et al. 1995, Malcolm, K. C., et al. 1994, Ohguchi, K., et al. 1995, Siddiqi, A. R., et al. 1995), and partially purified extracts of rat and porcine brain, human placenta and porcine aortic endothelial (PAE) cells (Cross, M. J., et al. 1996, Kuribara, H., et al. 1995, Singer, W. D., et al. 1995, Vinggaard, A. M., et al. 1997). Furthermore the addition of recombinant forms of the Rho and the related Rho-family proteins Rac1 and Cdc42 to membranes previously treated with Rho-GDI (to extract Rho family proteins) resulted in restoration of GTPγS-stimulation of PLD (Kwak, J. Y., et al. 1995, Malcolm, K. C., et al. 1994, Siddiqi, A. R., et al. 1995). Recombinant Rac was also able to mediate activation of PLD by treatment of fibroblasts with epidermal growth factor (EGF) (Hess, J. A., et al. 1997), and recombinant Cdc42 is an activator of the partially purified PLD activity from porcine brain (Singer, W. D., et al. 1995). Rho was defined as an activator of the cloned human PLD1 enzyme by Hammond *et al.* (Hammond, S. M., et al. 1997). Recent work on the M_3 receptor stably expressed in HEK cells has shown that carbachol-stimulation of PLD in these cells is mediated in part by Rho proteins. Rho can be inactivated by monoglucosylation by *Clostridium difficile* toxin B (Just, I., et al. 1994, Just, I., et al. 1995), and ADP-ribosylation by *Clostridium botulinum* C3 exoenzyme (Aktories, K., et al. 1988, Aktories, K., et al. 1987, Paterson, H. F., et al. 1990, Ridley, A. J. and A. Hall 1992, Sekine, A., et al. 1989). The activation of PLD by the M_3 receptor expressed in HEK cells and direct stimulation by GTPγS and AlF_4^-, was sensitive to both toxin B and C3 exoenzyme (Schmidt, M., et al. 1996, Schmidt, M., et al. 1996). As shown in Figure 3.4, the Rho inhibitor C3 exoenzyme significantly ($p<0.05$) reduced the carbachol- but not

thrombin-stimulated PLD response, compared to the agonist stimulated response. The wtRhoA construct was able to increase the PLD response elicited by a low concentration of carbachol (20 μM). However the wtRhoA construct had no analogous effect on the PLD activity stimulated by a low concentration of thrombin (0.2 Units/ml). Moreover the negative RhoA construct inhibited the carbachol- but not the thrombin-induced PLD activity. Therefore the M_3, but apparently not the thrombin receptor can couple to PLD through both ARF and RhoA in 1321N1 cells, as already proposed for stably expressed M_3 receptors in HEK cells (Rümenapp, U., et al. 1995, Schmidt, M., et al. 1996, Schmidt, M., et al. 1996).

Close association of receptor with G proteins

The interaction of receptor and G protein is a close one, and work done using antibodies directed against G proteins, reconstitution experiments, synthetic receptor intracellular loop peptides, mutation of i2 and i3 and receptor-G protein crosslinking experiments loops all contribute to the evidence for a close interaction (reviewed in (Bourne, H. 1997, Gudermann, T., et al. 1996, Savarese, T. M., et al. 1992, Wess, J. 1997)). For example the carboxy terminal tail of the α subunit of transducin (G_t), especially the last 5 amino acids interact directly with rhodopsin (Conklin, B. R., et al. 1993, Dratz, E. A., et al. 1993, Garcia, P. D., et al. 1995, Hamm, H. E., et al. 1988). Also a peptide representing the Ci3 of the α_2-adrenergic receptor could be crosslinked to the alpha and beta subunits of G_o (Taylor, J. M., et al. 1994). This direct association is further supported by the cross-linking of the agonist bound VIP_1 receptor to G_s (Kermode, J. C., et al. 1992). Immunoprecipitation approaches have also shown a direct interaction of the α_{2a}-adrenergic, 5-HT_{1A} receptors with $G\alpha_i$ (Okuma, Y.

and T. Reisine 1992, Raymond, J. R., et al. 1993) and the μ and δ opioid receptors with $G\alpha_o$ (Georgoussi, Z., et al. 1995). In our case the co-precipitation of [^{3}H]NMS binding sites with ARF1/3 and RhoA, supports the close interaction of the M_3 receptor with these small G proteins (Figure 3.5). There was no specific association of thrombin receptor with ARF1/3 and RhoA, as shown in Figure 3.6, by the lack of recovery of thrombin receptor ligand (Feng, D. M., et al. 1995) i.e. [^{125}I]TRP binding sites in ARF1/3 and RhoA immunoprecipitates. The association of the M_3 receptor with ARF1/3 and RhoA was dependent on pre-exposure of 1321N1 cells to carbachol (100 μM, for 10 mins). The inability of the polyclonal ARF1/3 and RhoA antibodies to co-precipitate [^{3}H]NMS binding sites, from 1321N1 cells not exposed to agonist, is consistent with the reports of the translocation of ARF1/3 and RhoA from the cytosol to the cellular membranes in response to agonist (Houle, M. G., et al. 1995, Malcolm, K. C., et al. 1996, Rümenapp, U., et al. 1995). We infer that the redistribution of the ARF and Rho proteins allows the receptor to newly interact with them and perhaps other cofactors. In addition, occupation of the receptor by agonist may be a prerequisite for conformational changes permissive to docking of ARF/Rho (or their intermediaries). The incubation of the 1321N1 cells with 100 μM BFA, during the priming process, reduced the quantity of [^{3}H]NMS sites co-precipitated by ARF1/3 polyclonal antibodies by 74 ± 19% (data not shown). The effect of BFA will be to block the exchange of guanine nucleotides on ARF, and this inhibition will prevent the association of soluble ARF proteins with membranes (Donaldson, J., et al. 1992, Helms, J. B. and J. E. Rothman 1992, Randazzo, P. A., et al. 1993). This may prevent ARF interacting with other cofactors and the receptor, or alternatively directly interacting with the receptor. The co-precipitation of the M_3 receptor could be dramatically

reduced to control levels by the inclusion of excess ARF1/3 or RhoA peptide antigen, demonstrating that the [^{3}H]NMS binding sites were associated with authentic ARF and Rho proteins. The lack of specific [^{125}I]TRP binding in RhoA immunoprecipitates, is interesting as the employment of Rho proteins, downstream of thrombin receptor activation is widely reported. Other routes of Rho activation may of course be involved in these circumstances, which do not relate to the relatively direct docking processes seen for example with the M_3 receptor. Thrombin-stimulation of NIE-115, NG108-15 cells and astrocytes causes neurite retraction and rounding of the cell body (Jalink, K. and W. H. Moolenaar 1992, Jalink, K., et al. 1994), Moreover in NIE-115 and NG108-15 cells, thrombin has been shown to activate Rho, and the reported thrombin-evoked morphological changes were Rho-dependent and blocked by treatment of these cells with C3 exoenzyme (Jalink, K. and W. H. Moolenaar 1992, Jalink, K., et al. 1994). The Rho family of proteins are known to regulate the morphological responses and actin cytoskeleton, including formation of actin stress fibre and focal adhesions (Hall, A. 1992, Ridley, A. J. and A. Hall 1992). More importantly thrombin was found to induce an increase in DNA synthesis and the rounding of 1321N1 cells, a reversal of their normally stellate morphology, moreover carbachol, LPA and bradykinin-stimulation of 1321N1 cells did not cause cell rounding (Majumdar, M., et al. 1998). The rounding of 1321N1 cells and the increase in DNA synthesis was completely abolished by treatment of cells with C3 exoenzyme. It is apparent that both the thrombin and M_3 receptors can couple to Rho, however only the M_3 receptor employs a close association with Rho, and ARF, in the activation of PLD. The coupling of the thrombin receptor to Rho in 1321N1 cells is upstream of increases in DNA synthesis and cell rounding. Recently the coupling of

the thrombin receptor to the increases in gene expression, increases in DNA synthesis and mitogenesis in 1321N1 cells was shown to be downstream of the receptor coupling to G_{12} (Aragay, A. M., et al. 1995, Post, G. R., et al. 1996). Similarly the thrombin receptor in platelets interacts with G_{12} and G_{13} (Offermans, S., et al. 1994). The G_{12} and G_{13} proteins have been shown to interact specifically with the mammalian Rho guanine nucleotide exchange factor Lsc/p115^{RhoGEF}2, and furthermore G_{13} stimulates GDP/GTP exchange on Rho in the presence of Lsc/p115^{RhoGEF} (Hart, M. J., et al. 1998, Kozasa, T., et al. 1998). The G_{12} protein can inhibit the stimulation of Rho GDP/GTP exchange by G_{13}, and may represent a mechanism by which GPCRs can moderate the level of Rho activation (Hart, M. J., et al. 1998). It is also possible that G_{12} stimulates another closely related Rho guanine nucleotide exchange factor. Thus the thrombin receptor may interact closely with G_{12} and G_{13}, leading to a subsequent increase in Rho activity and the rearrangement of the actin cytoskeleton. Similarly the coupling of the TXA_2 receptor to G_{12} and G_{13} in platelets suggests similar signalling characteristics for both $DPX_{2-3}Y$ containing receptors (Offermans, S., et al. 1994).

The close association of the M_3 receptor with small G proteins was further investigated using the immunodetection of ARF and Rho in the immunoprecipitates produced using polyclonal antiserum raised against the M_3 receptor (Wall, S. J., et al. 1991). The authenticity of the ARF and Rho proteins in immunoprecipitates produced using polyclonal antisera against ARF1/3 (Martin, A., et al. 1996), or RhoA (Laudanna, C., et al. 1996) was confirmed by immunoblotting of polyclonal immunoprecipitates with monoclonal antibodies (Figure 3.7). As shown in Figure 3.8 the immunoprecipitation of authentic ARF and Rho from

the membrane fraction of 1321N1 cells, using M_3 receptor antiserum, was dependent on the exposure of cells to carbachol. Similarly, recovery of authentic ARF1/3 and RhoA from membrane-derived immunoprecipitates, directed against those proteins was greatly enhanced by carbachol priming (Figure 3.7, and data not shown). This would be consistent with the hypothesis that carbachol priming allows an essential translocation of ARF and Rho to the membrane as reported previously (Houle, M. G., et al. 1995, Malcolm, K. C., et al. 1996, Rümenapp, U., et al. 1995). The use of non-immune reagents did not co-precipitate immunoreactive ARF or Rho, and antibody blocking with excess peptide antigen confirmed the specificity of the interactions.

Close association of receptors and G proteins in *ex vivo* tissue

The body of work described here suggests that in response to agonist, the GPCRs that contain the conserved $NPX_{2-3}Y$, but not the $DPX_{2-3}Y$ motif, activate PLD via a close association with the small G proteins ARF and Rho. A prototypical $NPX_{2-3}Y$ receptor, the M_3 muscarinic receptor, but not the $DPX_{2-3}Y$-containing thrombin receptor, can form a close association with the small G proteins ARF and Rho. The presence of the $NPX_{2-3}Y$ sequence within the TMD 7 is suggested as a key determinant, controlling the selectivity of this coupling. The importance of the $NPX_{2-3}Y$ motif in the close interaction of small G proteins was further confirmed using the AT_1 receptor which is natively expressed in lactotrophs of the male rat anterior pituitary gland. The AT_1 receptor activates PLD in a BFA-sensitive manner, consistent with the idea of an ARF-dependent mechanism being necessary. Moreover the immunoprecipitation of AT_1 receptors with polyclonal antibodies also co-precipitated immunoreactive ARF in an agonist-dependent manner, this

is shown clearly in Figure 3.10. The co-precipitation of ARF with anti-AT_1 receptor antiserum was specific for the AT_1 receptor, as the presence of excess blocking peptide, prevented the co-precipitation of ARF.

Selectivity of $NPX_{2-3}Y/DPX_{2-3}Y$ receptor interaction with G proteins

The interaction of the muscarinic M_3 receptor with the selection of G proteins in 1321N1 cells, and the modulation of receptor affinity for agonist and PLD activation was further investigated. The stimulation of cellular G proteins in digitonin-permeabilized 1321N1 cells with GTPγS, BeF_3^- or $GPPCH_2P$, decreased the receptor affinity for carbachol, thereby increasing the concentration of agonist required to displace 50% of the bound [^{3}H]NMS. GTPγS was the most potent agent at reducing the receptor affinity for agonist, and its effectiveness did not significantly change in control or agonist primed cells. BeF_3^- was more effective at increasing the IC_{50} for carbachol, compared to $GPPCH_2P$ in control treated cells. However once pre-exposed to 20 μM carbachol, $GPPCH_2P$ was more effective than BeF_3^- at decreasing receptor affinity for agonist, as shown in Table 3.1a. The stimulation of G proteins with GTP analogues, is generally considered to prevent their interaction with receptors and convert the receptor to a state with low affinity for agonist. The priming of 1321N1 cells appears to increase the receptor interaction with $GPPCH_2P$-activated G proteins, and not those sensitive to BeF_3^-. The BeF_3^- induced reduction in receptor affinity for carbachol was attenuated in the membranes of cells that had been primed with agonist. In contrast, priming potentiated the ability of $GPPCH_2P$ to decrease the receptor affinity for carbachol. These results suggest that once exposed to agonist the receptor increases its recruitment of $GPPCH_2P$- but not the BeF_3^--sensitive G proteins and the association with receptor restores a high

affinity receptor state, which can be observed by its uncoupling in the presence of relevant GTP analogues. GTPγS is a potent activator of both trimeric and small G proteins, whereas fluoride complexes of aluminium have been shown to activate the G_s, G_i, and G_q, G proteins (Guillon, G., et al. 1986, Howlett, A. C., et al. 1979, Katada, T., et al. 1984, Sternweis, P. C. and A. G. Gilman 1982). However fluoride complexed to aluminium (or beryllium, a more rigid isostere), have been proposed to be an activator of trimeric but not small G proteins (Bigay, J., et al. 1987, Kahn, R. A. 1991). Nonetheless, AlF_4^-, has recently been shown to bind to the GDP-bound form of Ras, Ran, Rap and Cdc42, although whether activation ensued was not shown, and effects on ARF were not also not reported (Ahmadian, M. R., et al. 1997). Analogues of GTP containing βγ-methylene groups such as $GPPCH_2P$, are known to be potent activators of ARF, Rab5 and other small G proteins, although they are ineffective stimulators of trimeric G proteins and Ras (Gill, D. M. and J. Coburn 1987, Hoffenberg, S., et al. 1996). Therefore it seems likely that priming recruits small G proteins to the receptor and this might be the mechanism by which the M_3 receptor is maintained in a high affinity state. The distribution of trimeric G proteins at the plasma membrane would allow them to be activated first by the ligand bound receptor. The translocation of small G proteins from the cytosol to the receptor may represent a secondary effect initiated by of agonist binding.

The activation of PLD was also modulated by the incubation of permeabilized 1321N1s with GTP analogues. GTPγS, BeF_3^- and $GPPCH_2P$, stimulated a 150 ± 9, 115 ± 14 and 48 ± 8% increase in PLD activity in 1321N1 cells not previously exposed to agonist. The direct activation of PLD by BeF_3^- was approximately 2.4 times the response elicited by

$GPPCH_2P$. This may reflect a greater capacity of the trimeric G proteins to activate PLD, independent of receptor stimulation. The $GPPCH_2P$- but not the BeF_3^--induced increase in [^{3}H]PtdBut, was inhibited by BFA and C3 exoenzyme, demonstrating the involvement of ARF and Rho, and consistent with the preference of $GPPCH_2P$ for small G proteins. Pre-exposure of the 1321N1 cells to 100 μM carbachol, was sufficient to cause detectable changes in the characteristics with which the cellular complement of PLD was regulated by G proteins (Table 3.1b). The GTPγS-induced activation of PLD did not significantly vary from the activity in unprimed cell, consistent with the activation of all G proteins. However the BeF_3^--stimulated PLD activity decreased by 32 ± 9% in primed, compared to unprimed cells. Conversely the $GPPCH_2P$-induced PLD activity increased by 56 ± 7% in primed 1321N1 cells. Consequently these results are in agreement with the hypothesis that exposure to agonist leads to the increased involvement of small but not trimeric G proteins in mechanisms leading to the activation of membrane PLD. The drastic reduction by BFA and C3 exoenzyme, of the $GPPCH_2P$-induced PLD response in primed cells, confirms a key role for ARF and Rho in these responses.

Potentially relevant structural motifs in receptors

The $NPX_{2-3}Y$- and not the $DPX_{2-3}Y$-containing receptors thus appear to form functional interactions with ARF and Rho in the activation of PLD. The conservation of the $N/DPX_{2-3}Y$ sequence across the evolution of GPCRs points to an important role in the functioning of the receptor. A recent model of the aminergic GPCRs suggests that the $N/DPX_{2-3}Y$ sequence is ideally placed to receive a signal from agonist-induced conformational changes in the ligand binding domain (Donnelly, D., et al.

1994, Findlay, J. B. C., et al. 1993). The asparagine and tyrosine residues conserved within the TMD 7 motif are proposed to participate in a hydrogen- (H) bonding network, involving other TMDs (Ballesteros, J., et al. 1998, Fanelli, F., et al. 1995, Hunyady, L., et al. 1995, Konvicka, K., et al. 1998, Liu, J., et al. 1995, Oliveira, L., et al. 1994, Perlman, J. H., et al. 1996, Scheer, A., et al. 1996). The role of the aspartate residue of the $DPX_{2-3}Y$ motif in H-bonding to other amino acids has not been probed, although in mutants of the β_2 and TRH receptor, aspartate could in part substitute for asparagine with regard to receptor activation (Barak, L. S., et al. 1995, Perlman, J. H., et al. 1997). However replacement of this conserved asparagine with alanine in the TRH, β_2, AT_1 and $5\text{-}HT_{2A}$ receptors significantly inhibited or abolished appropriate second messenger responses (Barak, L. S., et al. 1995, Hunyady, L., et al. 1995, Perlman, J. H., et al. 1997, Sealfon, S. C., et al. 1995). Therefore the conservative nature of the aspartate substitution in the TRH and β_2 receptors fits the idea that aspartate can fulfil an asparagine's H-bonding commitments.

Inter-helical interactions and receptor activation

Molecular models for the arrangements of GPCRs have been described by Baldwin *et al.*, based upon the conservation of amino acids, hydropathy and alignment of multiple GPCR sequences, and using the low resolution map for rhodopsin described by Schertler *et al.* as a template (Baldwin, J. M. 1993, Schertler, G. F. X., et al. 1993). The orientation of the transmembrane helices derived from these modelling experiments place TMDs 1, 2, 3 and 7 in close proximity to each other. Moreover, molecular modelling of various GPCRs has consistently implicated a network of hydrogen- (H) bonding between TMDs 1, 2, 3 and 7 in the regulation of receptor conformation (Fanelli, F., et al. 1995, Konvicka, K., et al. 1998,

Oliveira, L., et al. 1994, Scheer, A., et al. 1996, Scheer, A., et al. 1997) Furthermore, these models are in agreement with the experimental evidence from mutagenesis studies pointing to helix-helix interactions in the AT_1, TRH, M_3 and α_{1B} adrenergic receptors (Hunyady, L., et al. 1995, Liu, J., et al. 1995, Perlman, J. H., et al. 1997, Scheer, A., et al. 1996), and numerous other receptors reviewed by Baldwin *et al.* (Baldwin, J. M. 1993, Baldwin, J. M. 1994). The amino acids involved in the proposed H-bonding network are numbered according to a scheme described by Ballesteros and Weinstein (Ballesteros, J. A. and H. Weinstein 1995). The scheme consists of a digit identifying the transmembrane domain (1 to 7), and the number describing the position in the domain relative to the most conserved amino acid in that helix, which is given the number 50. The amino acids involved include the most highly conserved amino acids N1.50, D2.50, D3.49, R3.50, N7.49 and Y7.53. The molecular modelling of the M_3, α_{1B}-adrenergic, TRH and 5-HT_{2A} receptors, using molecular dynamic or other receptor conformational simulations of the H-bonding system implicates a group of highly conserved polar amino acids in the regulation of receptor function (Fanelli, F., et al. 1995, Konvicka, K., et al. 1998, Perlman, J. H., et al. 1997, Scheer, A., et al. 1996). These investigators propose hydrogen bonding interactions between the amino acids, N1.50, D2.50, R3.50, N7.49, and Y7.53 in these receptors. The N7.49 and Y7.53 residues are the asparagine and tyrosine residues that are part of the $NPX_{2-3}Y$ sequence. The conserved N1.50 is proposed to H-bond with D2.50 and N7.49 in the α_{1B}, TRH and 5-HT_{2A} receptors, acting as a type of polar bridge (Konvicka, K., et al. 1998, Perlman, J. H., et al. 1997, Scheer, A., et al. 1996), In the 5-HT_{2A} receptor modelling also suggests a H-bond interaction of between the N1.50 and Y 7.53 (Konvicka, K., et al. 1998). In the α_{1B} and M_3 receptors, molecular dynamic

simulations of receptor conformation propose that R3.50 H-bonds to D1.50 (Fanelli, F., et al. 1995, Scheer, A., et al. 1996). The N1.50, D2.50, N7.49, and Y7.53 conserved amino acid residues form a "polar pocket", highlighted previously in modelling studies (Fanelli, F., et al. 1995, Oliveira, L., et al. 1994, Trumpp-Kallmeyer, S., et al. 1992), in which the conserved amino acid R3.50 sits. The residue R3.50 is part of the highly conserved DRY sequence at the beginning of the second intracellular loop (i2), mutagenesis studies have shown that the conserved DRY motif is important in the receptor coupling to G protein (Gudermann, T., et al. 1996, Probst, W. C., et al. 1992, Savarese, T. M. and C. M. Fraser 1992, Wess, J. 1997). Once agonist binds, the conserved aspartate (D3.49), of the DRY motif is protonated and the polar pocket formed by the conserved amino acids is perturbed, resulting in the displacement of the conserved R3.50. The shift in the position of R3.50 is proposed to allow amino acids in i2 and i3 to achieve a correct conformation for G protein coupling (Fanelli, F., et al. 1995, Scheer, A., et al. 1996, Scheer, A., et al. 1997). Studies with wild-type and mutant rhodopsin receptors are consistent with the proposal that the neutralisation of the acidic amino acid (Glu or Asp) preceding the conserved R3.50, is required for the adjacent R3.50 side chain to achieve an orientation that is suitable for receptor interactions with G proteins (Acharya, S. and S. S. Karnik 1996, Arnis, S., et al. 1994, Scheer, A., et al. 1996, Scheer, A., et al. 1997). In agreement with this concept, mutational studies have shown that replacement of the conserved Glu/Asp in the α_{1B} receptor or rhodopsin with neutral amino acids, leads to receptors that are constitutively active (Arnis, S., et al. 1994, Cohen, G. B., et al. 1993, Scheer, A., et al. 1996, Scheer, A., et al. 1997). The replacement of a conserved asparagine with serine in the M_3 receptor, which also causes the spatial displacement of the R3.50, was found to

activate the receptor (Blüml, K., et al. 1994, Fanelli, F., et al. 1995). These findings are in agreement with the hypothesis of *Oliveira et al.* that binding of an agonist may initiate a conformational change which shifts the side chain of the conserved R3.50, and favours receptor-G protein coupling (Oliveira, L., et al. 1994). Differences in the H-bonding behaviour of the aspartate residue at position 7.49, compared to the asparagine are likely to greatly effect the movement of the arginine of the DRY motif. Therefore the 5% of class I GPCRs which carry the $DPX_{2-3}Y$ motif, may exhibit an altered maintenance of the H-bonding pattern and the ionic environment around the conserved R3.50.

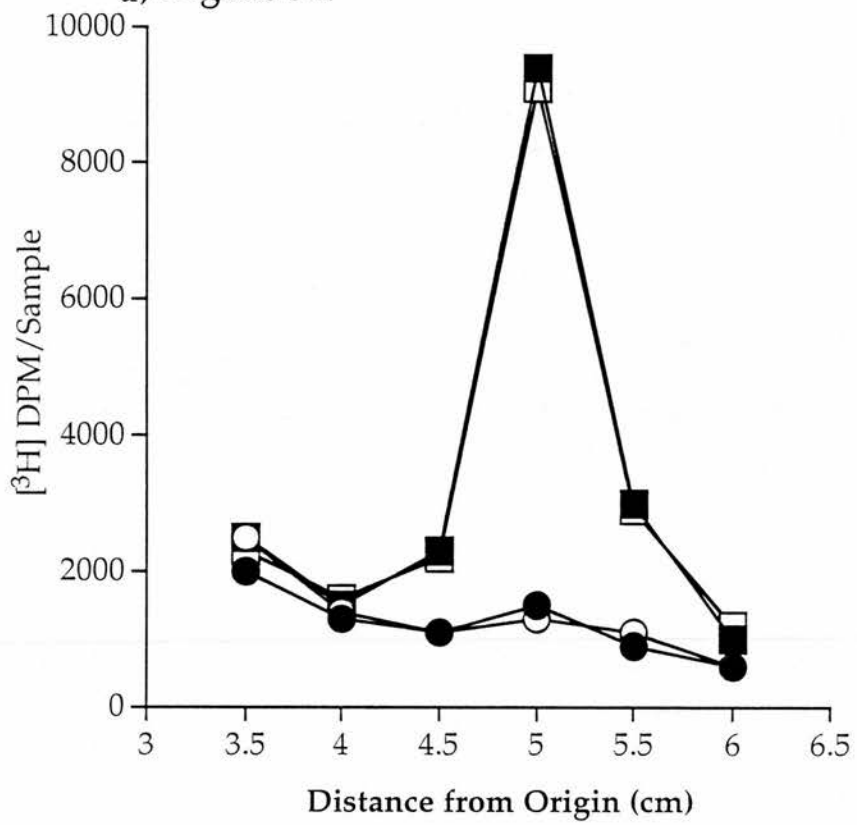
Further studies with the M_3 receptor demonstrate the importance of the proline residue within the $NPX_{2-3}Y$ motif. Wess *et al.*, show that the mutation of the proline residue to alanine, reduced expression of the mutant M_3 receptor by 35 times, compared to wild type receptor. This suggests that the conserved proline residue may play an important role in inducing or maintaining a proper protein fold that allows efficient receptor translocation and insertion into the plasma membrane. Moreover the mutation also significantly inhibits the inositol phosphate hydrolysis stimulated by carbachol (Wess, J., et al. 1993). Mutation of the proline residue in the M_3 receptor as with any other receptor, will eliminate the kink in the α-helix that a proline residue will generate (Brandl, C. J. and C. M. Deber 1986, von Heijne, G. 1991, Williams, K. A. and C. M. Deber 1991). The presence of the proline kink appears to be essential for the receptor to couple to G proteins properly. Konvicka *et al.* propose that the Asn/Asp-Pro motif conserved in TMD 7 is required for the proper function of the receptor, by acting as helix-breaker and producing a flexible hinge that allows the TMDs to H-bond in the correct

manner (Konvicka, K., et al. 1998). The role of prolines in the induction of kinks in protein structure is well reported (Brandl, C. J. and C. M. Deber 1986, von Heijne, G. 1991, Williams, K. A. and C. M. Deber 1991). However the NP and/or DP motif is proposed to induce a pronounced Pro-kink, with which the receptor is able to enter into different patterns of H-bonding. This matches the prediction from both computational and empirical methodologies, based on satisfying the propensity of polar residues buried within soluble proteins to stabilise themselves by H-bonding to other polar residues (Konvicka, K., et al. 1998, McDonald, I. K. and J. M. Thornton 1994).

The N/DPX$_{2-3}$Y sequence is within an area of the receptor that is close to the G protein-coupling surface of the intracellular loops, and suggested by modelling studies to be ideally placed to receive signals from the ligand binding domain (Donnelly, D., et al. 1994, Findlay, J. B. C., et al. 1993). The negative effects of removing the conserved asparagine, proline and tyrosine residues at positions 7.49, 7.50, and 7.53 point to a role for them in maintaining the proper receptor conformation, and allowing the receptor to transduce extracellular signals productively. The activation of a receptor is proposed to involve the rotation of helices and relative opening the receptor intracellular surface, to expose domains of the receptor necessary for G protein coupling, such as the second and third intracellular loops (Farrens, D. L., et al. 1996, Luo, X., et al. 1994, Sheikh, S. P., et al. 1996, Wess, J. 1997). The close interaction of the NPX$_{2-3}$Y- but not the DPX$_{2-3}$Y-containing receptors with ARF and Rho proteins implicates the asparagine residue in TMD 7 in the regulation of a receptor conformation that, upon agonist binding can selectively couple to small G proteins (or potentially their intermediaries). The participation in the

proposed H-bonding network through conserved amino acid residues in TMDs 1, 2, and 3, would fulfil this regulatory role, and the pronounced flexibility generated by the Asx-Pro combination is proposed to make this physically possible (Konvicka, K., et al. 1998). The contrast in the G protein-coupling of the $NPX_{2-3}Y$ and $DPX_{2-3}Y$ receptors, and their activation of PLD, may be a result of differences in receptor conformation. The acidic nature of the aspartate side chain may result in an altered H-bonding pattern between TMDs, relative to the uncharged asparagine, and an associated conformational difference. The complete conservation of the $N/DPX_{2-3}Y$ sequence, and the high degree of conservation observed for the other polar amino acids within the transmembrane helices of the class I family, makes them unlikely as the final determinants of the wide variety of G protein coupling observed in this family. However, the diversity of receptor-G protein interactions, as determined by other domains of class I GPCRs, appear to share a fundamental regulation by the $N/DPX_{2-3}Y$ sequence.

a) Figure 3.1


10000
8000
6000
4000
2000
0
[3H] DPM/Sample
3
3.5
4
4.5
5
5.5
6
6.5
Distance from Origin (cm)

b)

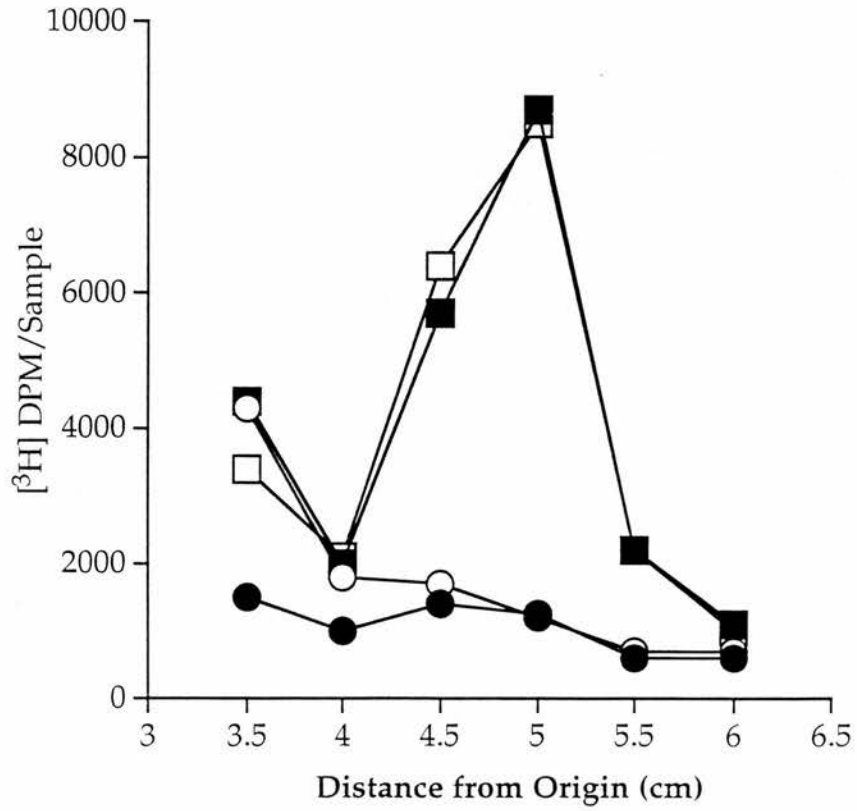

10000
8000
6000
4000
2000
0
[3H] DPM/Sample
3
3.5
4
4.5
5
5.5
6
6.5
Distance from Origin (cm)

Figure 3.2

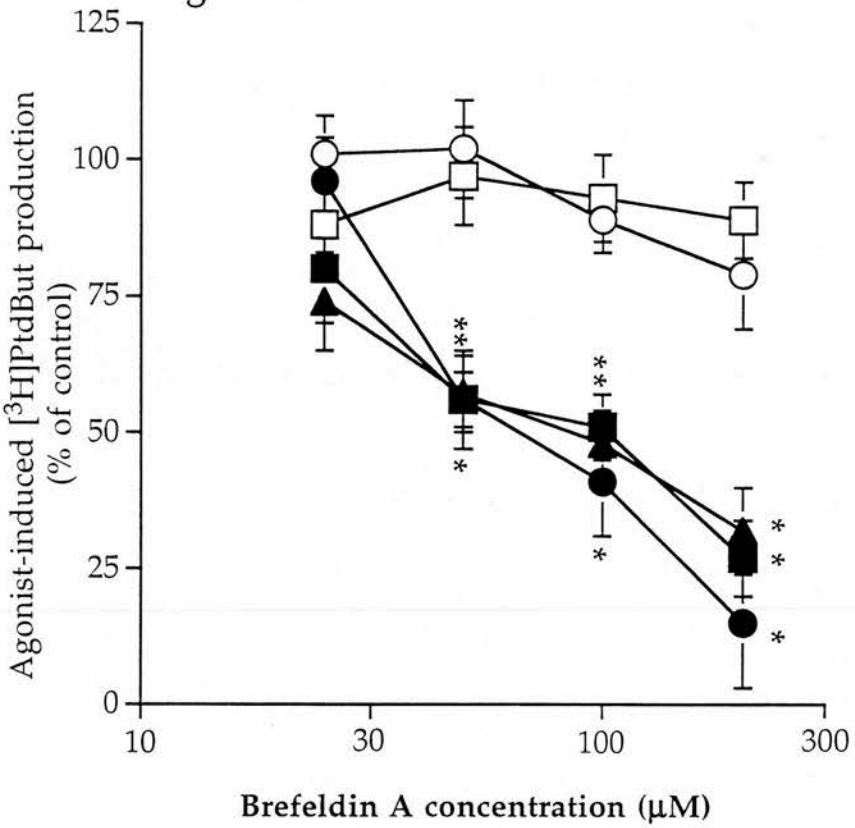

125
100
75
50
25
0
10
30
100
300
Agonist-induced [3H]PtdBut production
(% of control)
Brefeldin A concentration (μM)
*
**
**
*
**
*

Figure 3.3

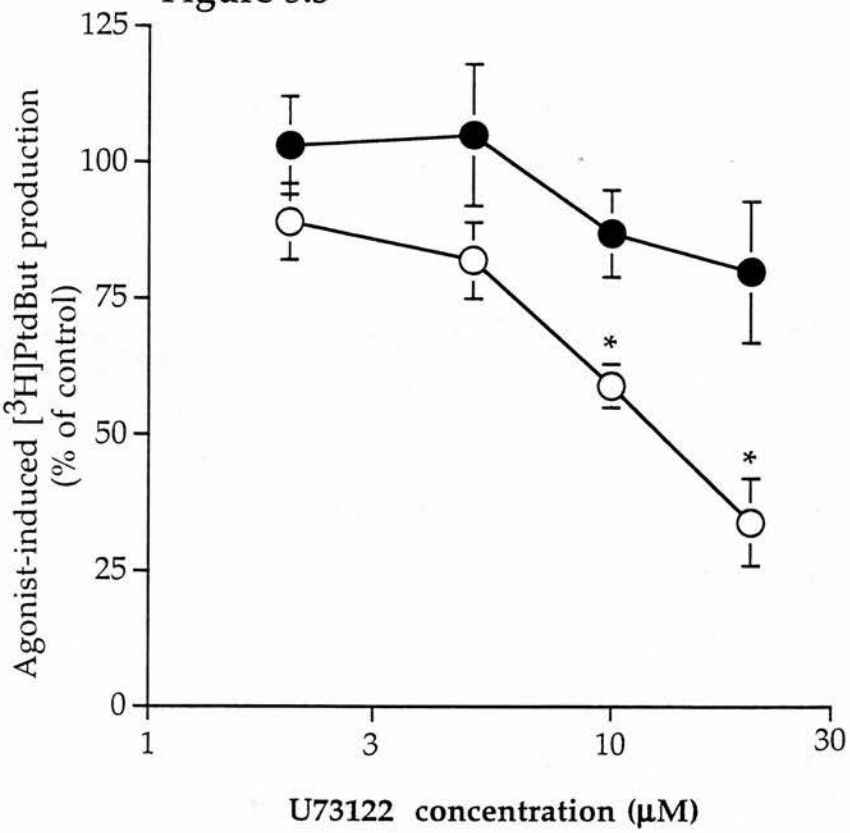

125
100
75
50
25
0
1
3
10
30
Agonist-induced [3H]PtdBut production
(% of control)
U73122 concentration (μM)
*
*

Figure 3.4

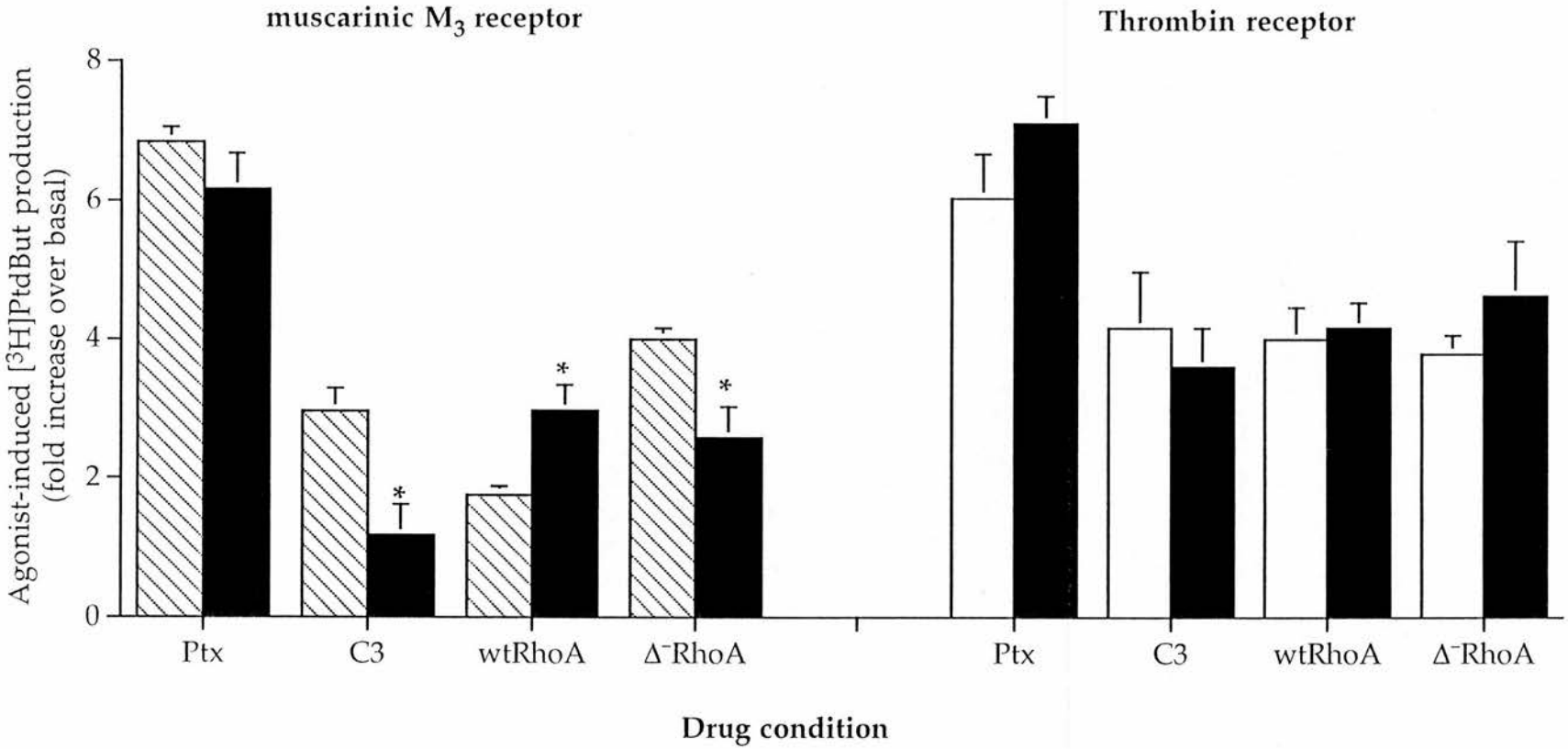

muscarinic M_3 receptor
Thrombin receptor
Agonist-induced [^{3}H]PtdBut production
(fold increase over basal)
8
6
4
2
0
*
*
Ptx
C3
wtRhoA
Δ⁻RhoA
Ptx
C3
wtRhoA
Δ⁻RhoA
Drug condition

Figure 3.5

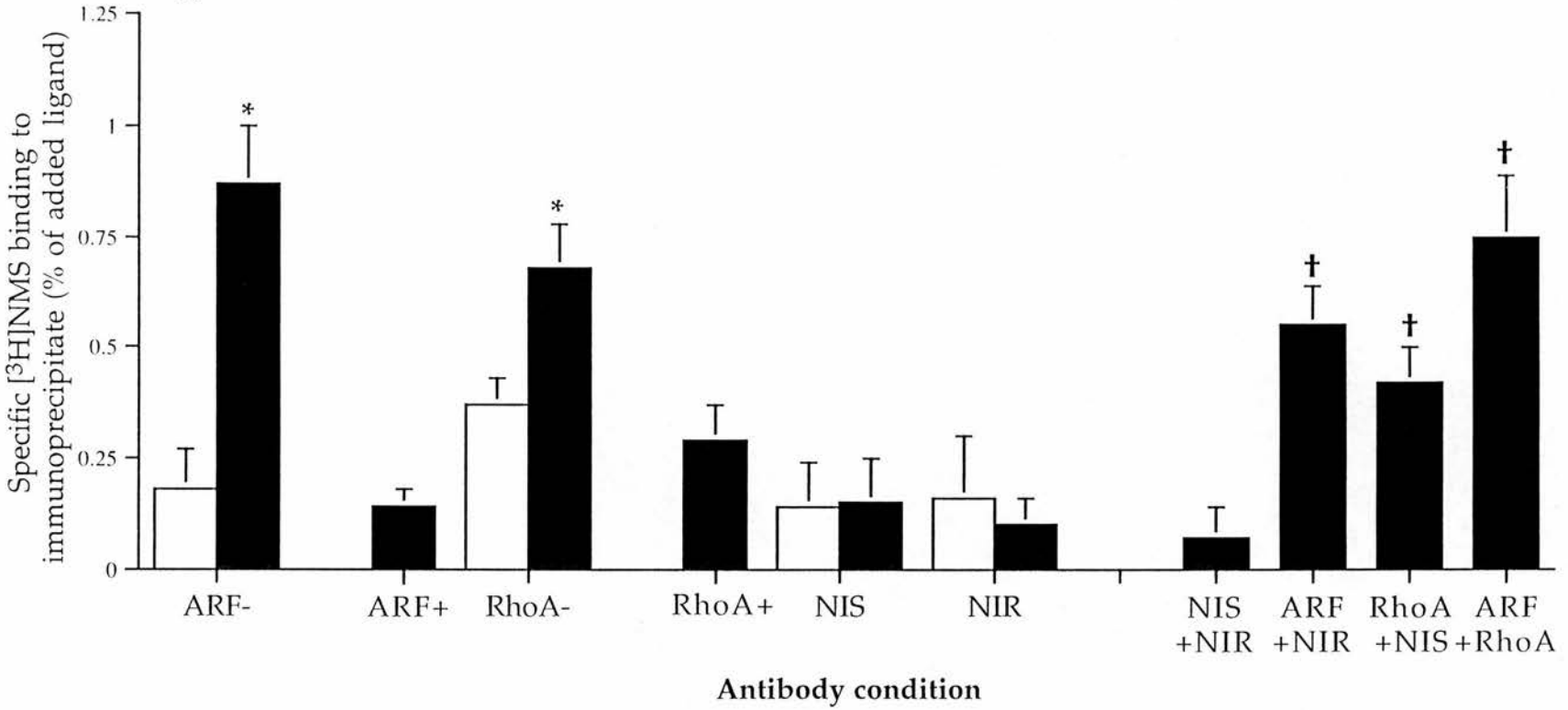

Specific [3H]NMS binding to immunoprecipitate (% of added ligand)
1.25
1
0.75
0.5
0.25
0
*
*
†
†
†
ARF-
ARF+
RhoA-
RhoA+
NIS
NIR
NIS +NIR
ARF +NIR
RhoA +NIS
ARF +RhoA
Antibody condition

Figure 3.6

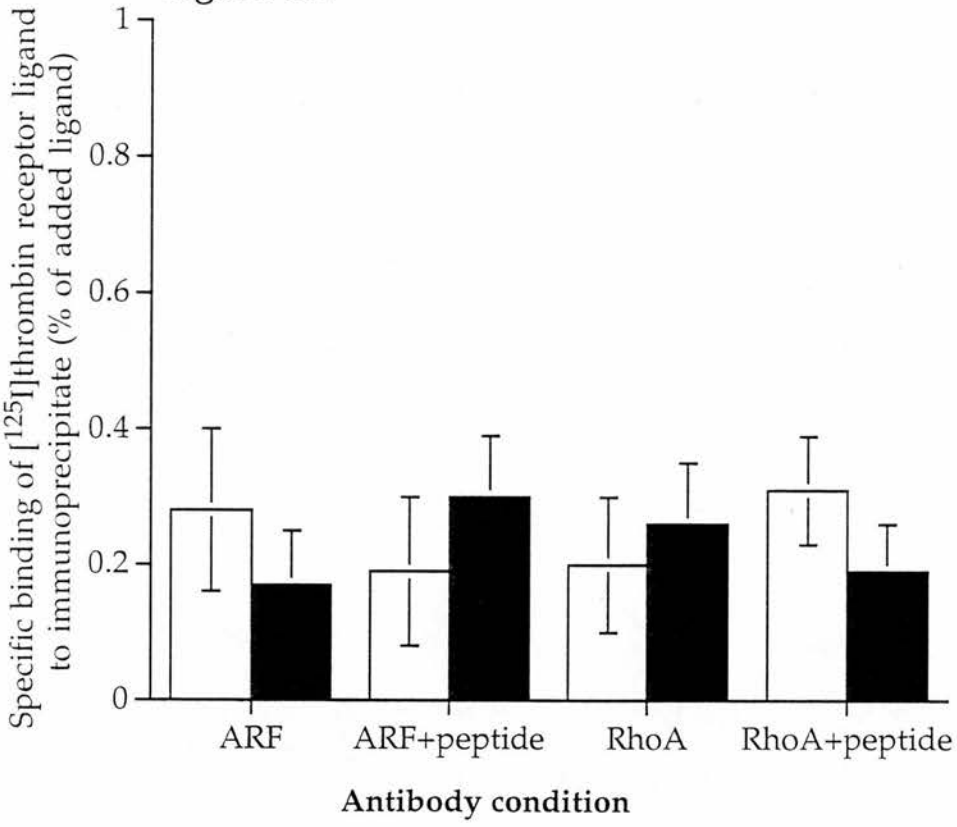

Specific binding of [125I]thrombin receptor ligand to immunoprecipitate (% of added ligand)
1
0.8
0.6
0.4
0.2
0
ARF
ARF+peptide
RhoA
RhoA+peptide
Antibody condition

Figure 3.7

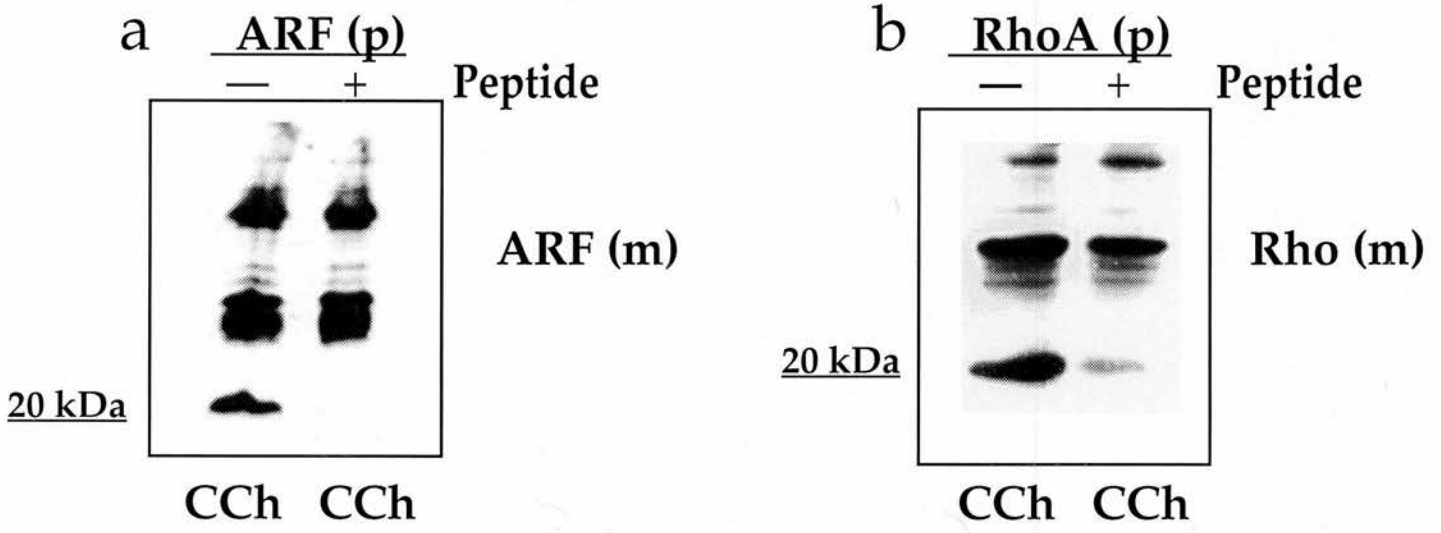

a
ARF (p)
—
+
Peptide
ARF (m)
20 kDa
CCh
CCh
b
RhoA (p)
—
+
Peptide
Rho (m)
20 kDa
CCh
CCh

Figure 3.8

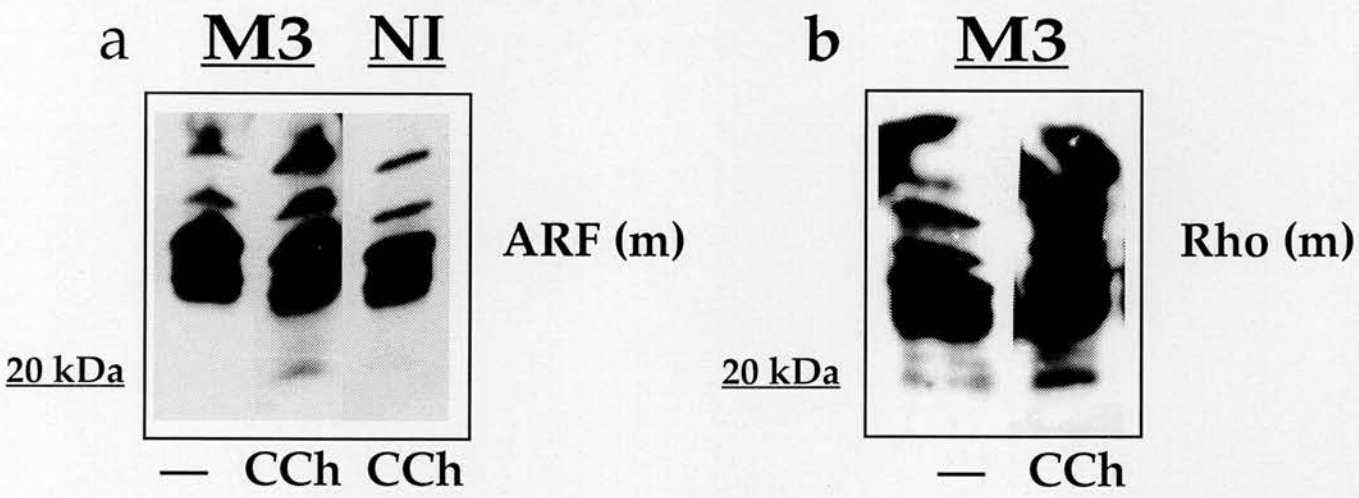


Figure 3.9

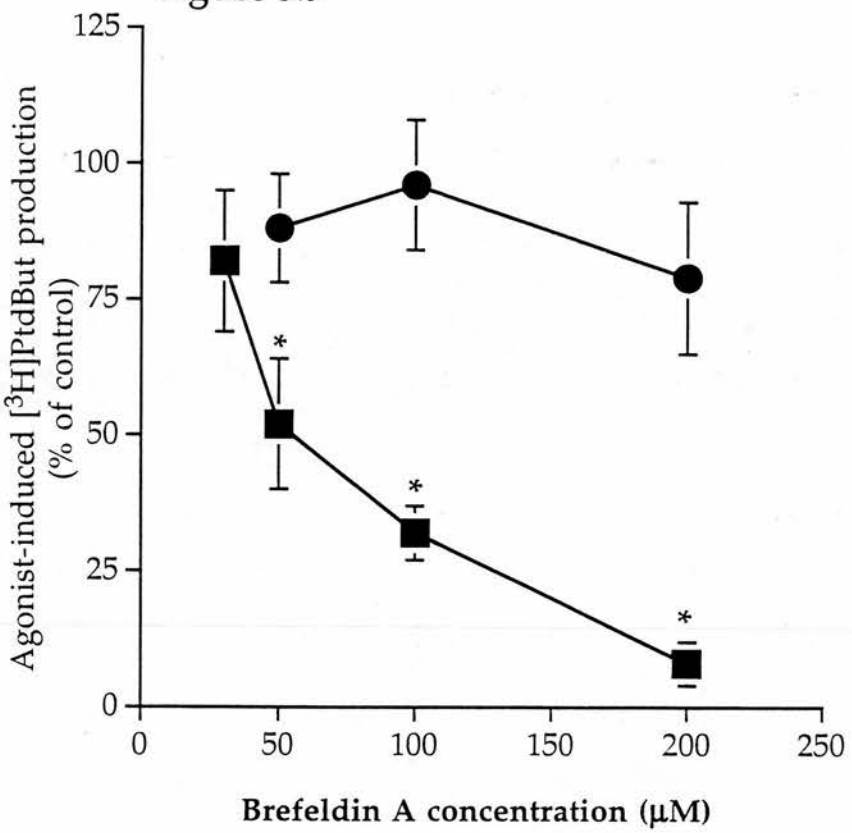

125
100
75
50
25
0
Agonist-induced [3H]PtdBut production
(% of control)
*
*
*
0
50
100
150
200
250
Brefeldin A concentration (μM)

Figure 3.10

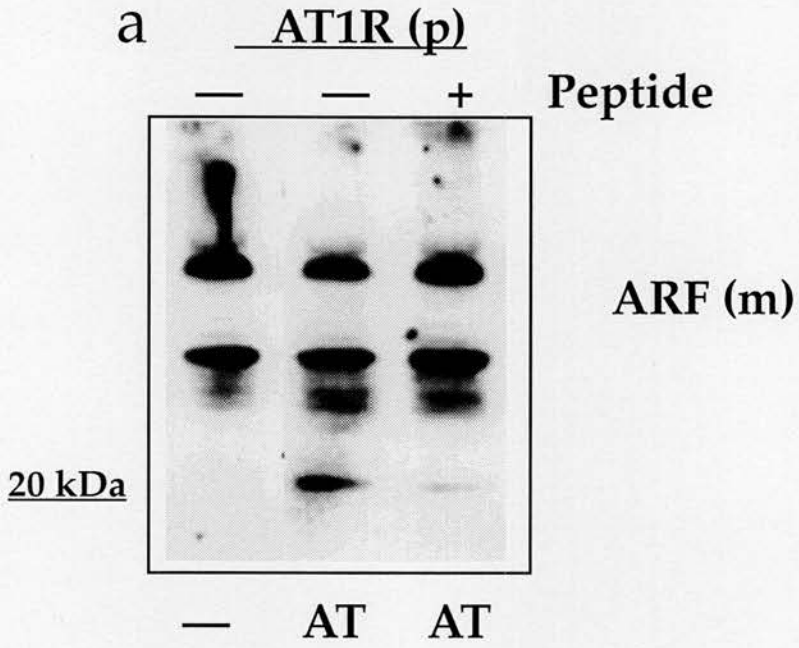


Table 3.1 **The effects of GTP analogues on agonist recognition by M_3 receptors and on PLD activation in 1321N1 cells**

Assay and treatment	GTP analogue		
	GTPγS	F^-	$GPPCH_2P$
(a) Carbachol affinity for membrane [^{3}H]NMS binding sites (fold increase in IC_{50})			
control	5.23 ± 0.97	4.06 ± 0.80	2.31 ± 0.34
carbachol-primed	4.48 ± 0.34	1.82 ± 0.31§	3.46 ± 0.40*
(b) Activation of PLD in digitonin-permeabilised cells (% increase over basal)			
control	150 ± 9	115 ± 14	48 ± 8
+ BFA	–	117 ± 9	31 ± 3†
+ C3 exoenzyme	–	95 ± 11	27 ± 5†
carbachol-primed	168 ± 10	78 ± 10§	110 ± 12*
+ BFA	–	69 ± 11	38 ± 9†
+ C3 exoenzyme	–	85 ± 9	23 ± 7†

GTPγS, F^- and $GPPCH_2P$ were present at 100 μM, 10 mM and 200 μM respectively. BFA and C3 exoenzyme were used at 100 μM and 4.8 μg/ml respectively. All values are means ± SEM, from 4-10 separate determinations. Statistically significant differences ($p<0.05$ Mann-Whitney U test) are indicated: * greater than corresponding unprimed; § less than corresponding unprimed; † reversal of GTP analogue effect.

CHAPTER 4

The activation of PLD by wild type and mutant GnRH and 5-HT_{2A} receptors

4.1 INTRODUCTION

The investigation of a role for the N/DPX$_{2-3}$Y sequence in the receptor coupling to PLD, described in Chapter 3, points to the importance of this motif in the gating of the receptor-G protein coupling mechanism. However the experiments relied on the use of different N/DPX$_{2-3}$Y motif-containing, G_q-linked receptors native to 1321N1 cells. Therefore it was in the context of very different receptor structures that the influence of the conserved sequence was being examined. Despite the strong correlation observed between the motif structure and utilisation of particular pathways, the involvement of receptor domains distinct from the N/DPX$_{2-3}$Y sequence cannot be excluded. In an attempt to unequivocally define the influence of the N/DPX$_{2-3}$Y motif in receptor activation of PLD through small G protein-dependent and independent pathways, we used the G_q-linked, wild type and mutant gonadotropin-releasing hormone (GnRH) and 5-hydroxytryptamine (5-HT/serotonin) $_{2A}$ receptors. The mutant form of these receptors had the residue at position 7.49 (the use of this numbering scheme was described by Ballesteros *et al*, and in Chapter 3 (Ballesteros, J. A. and H. Weinstein 1995)) replaced with the alternative aspartate or asparagine residue.

The mouse (Reinhart, J., et al. 1992, Tsutsumi, M., et al. 1992), rat (Eidne, K., et al. 1992, Kaiser, U. B., et al. 1992), human (Chi, L., et al. 1993, Karkar, S. S., et al. 1992), sheep (Brooks, J., et al. 1993, Illing, N., et al. 1992), and cow (Karkar, S. S., et al. 1993) GnRH receptors are unique amongst the class I GPCR family, in several respects, one of them being the complete absence of a carboxy (C) terminal tail. This region is probably involved in the mediation of desensitization and/or downregulation of some GPCRs

(Dohlman, H. G., et al. 1991). The C-terminal tail of most receptors contain consensus phosphorylation sites for PKC, protein kinase A (PKA), and the G protein-coupled receptor kinases (GRKs). Phosphorylation of the intracellular domains of GPCRs, including the C tail, by PKC, PKA and GRKs is implicated in the desensitization process (Hausdorff, W. P. 1990, Inglese, J., et al. 1993). The wtGnRH receptor expressed in αT3-1 or GH_3 cells showed no desensitisation of the receptor-stimulated InsP production over 90 mins (Davidson, J. S., et al. 1994, Sealfon, S. C., et al. 1992). The absence of the C-terminal tail allows the interpretation that any influence the $N/DPX_{2-3}Y$ motif has on G protein coupling is mediated through the intracellular loops (or transmembrane domains) and not the tail.

The wtGnRH receptor was chosen primarily because of the presence of an aspartate at position 318 (7.49, Asp318) in TMD 7, thereby generating a DPLIY sequence, and the receptor also has an asparagine at position 87 (2.50, Asn87) within the TMD 2. This arrangement of Asp and Asn residues appears to be unique and reciprocal to the situation found in the majority of class I GPCRs. Although the thrombin and TXA_2 receptors described in Chapter 3, possess the aspartate at position 7.49 in TMD 7 they do not have the atypical asparagine in TMD 2 (Hirata, M., et al. 1991, Vu, T. K. H., et al. 1991). Furthermore the human cytomegalovirus encodes a receptor which possesses the atypical Asn 2.50, but also has the canonical Asn at position 7.49 (Chee, M. S., et al. 1990). Mutagenesis and computer modelling studies of the wtGnRH receptor, reported by Zhou *et al*, point to the possible H-bonding of the Asn87 and Asp318 (Zhou, W. C., et al. 1994). The investigation of the $N/DPX_{2-3}Y$ motif in receptor G protein coupling and stimulation of PLD was carried out on the wtGnRH receptor

and single and double mutant receptors. The aspartate at 318 was replaced with an asparagine (Asn318 mutant) in the single mutant, and the double mutant also had the asparagine at position 87 replaced with an aspartate (Asp87-Asn318 mutant) (Zhou, W. C., et al. 1994). These mutations restore the arrangement of conserved Asp and Asn residues present in the majority of class I GPCRs (Probst, W. C., et al. 1992).

A wild type and mutant form of the human type 2A 5-HT receptor were used as comparisons for the experiments conducted using the wt and mutant GnRH receptors. The wt5-HT_{2A} receptor , like the majority of the class I GPCR family has a conserved asparagine in TMD 7 (7.49, Asn376) producing an NPLVY sequence (Saltzman, A. G., et al. 1991). This receptor also possesses the conserved aspartate at position 120 (2.50, Asp120) in TMD 2, and as with the mouse GnRH receptor, mutagenesis and computer simulation of the human 5-HT_{2A} receptor conformation upon activation, strongly implicates H-bonding between the conserved Asn376 and Asp120 residues (Sealfon, S. C., et al. 1995). The wt and a single mutant of the 5-HT_{2A} receptor, where the Asn376 was mutated to Asp376 were used as comparison with the sequence inversion in the GnRH receptors (and mutant) in tests on PLD activation.

In this way the wt and mutated forms of the GnRH and 5-HT_{2A} receptors allowed an investigation of the effect of either an $NPX_{2-3}Y$ or $DPX_{2-3}Y$ sequence on receptor stimulation of PLD. Furthermore, with the use of point mutants, any change in receptor coupling to small G proteins such as ARF and Rho can be attributed to the presence of the conserved asparagine or aspartate.

4.2 RESULTS

GnRH receptor signalling in response to agonist

The stimulation of PLC-mediated InsP production by the cloned wt and the mutant forms of the GnRH receptor expressed in COS 1, COS 7 and GH_3 cells has been reported elsewhere (Arora, K. K., et al. 1996, Awara, W. M., et al. 1996, Zhou, W. C., et al. 1994), and Figure 4.1 shows the time course for the stimulation of phosphoinositide breakdown by the wild type and mutant forms of the GnRH receptors. The wt, Asn318 and Asp87-Asn318 mutant GnRH receptor expressed transiently in COS 7 cells, mediated phosphoinositide breakdown (as stimulated by 100 nM) GnRH linearly over 40 minutes. The wild type receptor elicited a 4-5 fold increase in InsP production after 40 min compared to time zero. However the single and double mutant receptor were weaker in their coupling to PLC, with InsP responses at 40 min of, 39 ± 6% and 19 ± 5% of the wt receptor response. The weaker production of InsP by the mutant forms of the GnRH receptor was not a result of a diminished capability of COS 7 cells transfected with the mutant receptors to stimulate phosphatidylinositol 4,5-bisphosphate breakdown per se. Production of InsP by AlF_4^- (10 mM NaF and 30 μM $AlCl_3$) (presumed to cause stimulation of trimeric G proteins) was consistently stronger in COS 7 cells transfected with the Asn318 mutant GnRH receptor, at all time points than that mediated by the wtGnRH receptor. The weaker responses elicited by the mutant forms of the GnRH receptor, may be manifestations of the lower receptor number expressed on the plasma membranes of the transfected COS 7 cells and thus the maximal available binding sites for ligand (B_{max}). The B_{max} for the wt, Asn318 and Asp87-Asn318 mutant GnRH receptors was 589 ± 39, 230 ± 41 and 148 ± 36 fmol/mg protein

respectively as determined by membrane binding (Mitchell, R., et al. 1998).

The wtGnRH receptor also stimulated [^{3}H]PtdBut production linearly over the 40 min time course, with a 2-3 fold increase in PLD activation by the wtGnRH receptor after 40 min stimulation with 100 nM GnRH. However the Asn318 and Asp87-Asn318 mutant GnRH receptors induced a rapid increase in [^{3}H]PtdBut production that was 2.5 fold larger at 5 min, than the initial increase in PLD activity stimulated by the wt GnRH receptor (Figure 4.2). The concentration dependence for the activation of PLD by the wild type and the Asn318 mutant GnRH receptor was almost identical with EC_{50}s of 0.27 ± 0.10 nM and 0.16 ± 0.05 nM respectively, and the effect of GnRH on PLD activation by both receptors was maximal by 100 nM (Figure 4.3). This enhanced coupling to PLD by the mutant GnRH receptor is observed notwithstanding the poorer stimulation of InsP production and lower B_{max} described above. The PLD activity stimulated by both the Asn318 and Asp87-Asn318 mutant GnRH receptors, desensitised rapidly, in contrast to the linear PLC responses demonstrated by these receptors in Figure 4.1. The stimulation of trimeric G proteins with AlF_4^- caused non-desensitising stimulation of both PLC and PLD (Figures 4.1 and 4.2).

The enhanced stimulation and rapid desensitization of PLD seen with the mutant GnRH receptor may be due to an increased rate of receptor internalization and coupling to PLD. The presence of PLD at intracellular compartments has been described previously (Provost, J. J., et al. 1996, Whatmore, J., et al. 1996), and using protein tagging of the enzyme, PLD1 has been localised to the golgi, ER and lysosomal compartments (Brown, F. D., et al. 1998, Colley, W. C., et al. 1997, Hammond, S. M., et al. 1997).

However as shown in Figure 4.4 the inhibition of internalization using 200 μM monodansylcadaverine (MDC) and 30 μM monensin, increased rather than decreased the production of [^{3}H]PtdBut mediated by the wt and Asn318 mutant GnRH receptors, stimulated with 100 nM GnRH. MDC and monensin were shown to prevent the internalization of [^{125}I]buserelin binding in COS 7 cells expressing the wt and Asn318 mutant GnRH receptor (R. Mitchell, unpublished observations). MDC increased by 1.84 ± 0.15 and 3.52 ± 0.34 fold the production of [^{3}H]PtdBut by the wt and Asn318 mutant receptors respectively. Monensin increased the PLD activity by 1.84 ± 0.23 and 2.49 ± 0.16 fold, for the same 100 nM GnRH-stimulation of the wt and Asn318 mutant receptors. Although there is the possibility of non-specific effects of these internalization inhibitors at the concentrations used, these observations provided no evidence of a requirement for receptor internalization to increase the receptor coupling to PLD.

Involvement of PLC/PKC and ARF in the activation of PLD by the GnRH receptors

The observations reported in Chapter 3 suggested that GPCRs containing the $NPX_{2-3}Y$ sequence couple to PLD through ARF, whilst receptors with $DPX_{2-3}Y$ appear to utilise PLC in their stimulation of PLD. Therefore the activation of PLD by the wtGnRH receptor and the Asn318 mutant of the receptor was assessed with the ARF inhibitor BFA. The [^{3}H]PtdBut production stimulated by 100 nM GnRH in COS 7 cells expressing the Asn318 mutant GnRH receptor was significantly inhibited by BFA. By a concentration of 200 μM BFA, the Asn318 GnRH receptor-stimulated PLD activity was reduced by 72 ± 10%, compared to the control response with agonist alone, with an IC_{50} of 54 ± 8 μM. The wild type receptor

activated PLD in a manner that was resistant to the actions of BFA up to 200 μM (Figure 4.5). However the PLC inhibitor U73122 significantly reduced the wtGnRH receptor's activation of PLD with a IC_{50} of 11 ± 1 μM. The Asn318 mutant receptor stimulated PLD in a PLC-independent manner, as U73122 had no significant effect on the [^{3}H]PtdBut production induced by this receptor (Figure 4.6).

The stimulation of PLC can lead to an activation of PKC, and the activation of PLD by the wtGnRH receptor may be a consequence of this PKC activation. The activation of PLD by PKC, and the effect of BFA on this stimulation was studied by stimulation of PKC using the selective PKC inhibitor, GF 109203X (bisindolylmaleimide) (Toullec, D., et al. 1991), was employed to investigate the role of PKC in the stimulation of PLD by the wt and Asn318 mutant GnRH receptors. Figure 4.7 shows that the [^{3}H]PtdBut production stimulated by both receptors was inhibited by GF 109203X, with IC_{50} values for the wt and Asn318 GnRH receptors of 1.24 ± 0.44 and 1.29 ± 0.13 μM respectively, suggesting a role for PKC in the activation of PLD by both receptors. Further inhibitors of PKC such as the regulatory domain inhibitor calphostin C, were not tested, nor was the strategy of down-regulating PKC isoforms by prolonged preincubation with activating phorbol esters. The stimulation of PLD by direct activation of PKC, and the effect of BFA on this response are shown in Figure 4.8. PKC was stimulated using the calcium ionophore, ionomycin and phorbol 12,13-dibutyrate (PDBu). Calcium ions are necessary for the activation of the common PKC isoforms α, β1, β2 and γ. Moreover, PDBu mimics diacylglycerol, produced through the hydrolysis of PIP_2 by PLC, and activates PKC by interacting with a specific C2 lipid binding domain. Interestingly, in contrast with the results obtained using GF 109203X, the

activation of PLD by ionomycin and PDBu, as presented in Figure 4.8, was resistant to BFA, implying that the activation of PKC isoforms that bind calcium and/or possess the C2 lipid binding domain have no role in the pathway(s) leading from receptor to ARF in the activation of PLD. However these results do suggest that isoforms of PKC may be downstream or synergize with ARF in the activation of PLD.

Receptor association with small G proteins: ligand binding studies on ARF/RhoA immunoprecipitates

The association of the M_3 muscarinic but not the thrombin receptor with ARF and RhoA described in Chapter 3, suggests that ARF and Rho proteins can interact closely with $NPX_{2-3}Y$ and not $DPX_{2-3}Y$ receptors. The investigation of the wt and Asn318 mutant GnRH receptor's association with ARF and RhoA was similar to that described in Chapter 3, using anti-ARF1/3 (Martin, A., et al. 1996) and anti-RhoA (Laudanna, C., et al. 1996) polyclonal antibodies to immunoprecipitate specific GnRH receptor binding sites. The data in Figure 4.9 show that specific [^{125}I]buserelin binding sites were co-immunoprecipitated with ARF1/3 and RhoA in COS 7 cells expressing the Asn318 mutant but not the wt GnRH receptor. The presence of specific [^{125}I]buserelin binding in ARF1/3 and RhoA immunoprecipitates was dependent on the preincubation of cells with 100 nM GnRH for 15 min. Priming with GnRH significantly increases the level of [^{125}I]buserelin binding in ARF1/3 and RhoA immunoprecipitates 2.24 ± 0.24 fold and 3.25 ± 0.50 fold respectively, compared to unprimed controls ($p<0.05$, n = 4-8). COS 7 cells expressing the Asn318 mutant receptor, not preincubated with GnRH, demonstrated a level of [^{125}I]buserelin binding comparable to that achieved using control antibodies to $p21^{Ras}$ and extracellular signal-

related kinase (ERK) 1/2. The specific [^{125}I]buserelin binding associated with the ARF1/3 and RhoA immunoprecipitates in primed COS 7 cells expressing the Asn318 mutant GnRH receptor, could be reduced by 54 ± 9% and 73 ± 10% respectively, using excess ARF or Rho peptide antigen. This is analogous to the specific association of the M_3 receptor with ARF1/3 and RhoA, after exposure to agonist which was described in Chapter 3.

The differential modulation by GTP analogues of [^{125}I]buserelin binding to GnRH receptors

The dissociation rate of [^{125}I]buserelin from the wt and Asn318 mutant GnRH receptor was investigated, and the effects of GTPγS, GPPNHP, $GPPCH_2P$ and BeF_3^- on this parameter in digitonin-permeabilized COS 7 cells were studied. The wt and Asn318 mutant GnRH receptor demonstrated an initial decrease in [^{125}I]buserelin binding that appeared identical. Considering the way the experiment was set up with pre-loading of ligand at 4°C and the dissociation at 37°C this probably represents a temperature-dependent reduction in ligand binding (Perrin, M. H., et al. 1989). The results in Figure 4.10 display the modulation by nucleotides of the slower rate of ligand dissociation from the receptors occurring over the 15-50 min period after permeabilisation and transfer to 37°C. The slower rate for the dissociation of [^{125}I]buserelin was composed of a single linear element, with half-lives ($t_{1/2}$) of 22 ± 3 min and 26 ± 3 min, for the wt and Asn318 mutant receptor respectively. Treatment of digitonin-permeabilized COS 7 cells with increasing concentrations of GTPγS significantly increased the dissociation rate of [^{125}I]buserelin from both the wt and Asn318 mutant GnRH receptors, in an almost identical fashion (Figure 4.10a). The dissociation rate of [^{125}I]buserelin in the

presence of 100 μM GTPγS increased 1.72 ± 0.05 and 1.64 ± 0.06 fold compared to the control rate of the wt and Asn318 mutant receptors respectively. The GTP analogue GPPNHP was as effective as GTPγS in significantly reducing the $t_{1/2}$ for the dissociation of [^{125}I]buserelin from the wt and Asn318 mutant GnRH receptors (Figure 4.10b). The βγ–methylene analogue of GTP; $GPPCH_2P$, was able to significantly decrease the half life for the dissociation of [^{125}I]buserelin from the Asn318 mutant, but not the wt GnRH receptor. In the presence of 100 μM $GPPCH_2P$ the dissociation rate from the mutant receptor was increased by 1.58 ± 0.06 fold compared to control (Figure 4.10c). The incubation of permeabilized COS 7 cells with fluoride ions in the presence of beryllium produced the reciprocal result to that produced with $GPPCH_2P$. The half life for [^{125}I]buserelin dissociation from the wtGnRH receptor was significantly reduced by increasing concentrations of BeF_3^- (Figure 4.10d). The $t_{1/2}$ of [^{125}I]buserelin dissociation from the mutant receptor was not significantly affected. The involvement of ARF in the modulation of the ligand dissociation rate by the GTP analogues was investigated using brefeldin A. COS 7 cells were pretreated with increasing concentrations of BFA and then stimulated with 100 μM GTPγS. As shown in Figure 4.10a, GTPγS dramatically reduced the half life of [^{125}I]buserelin dissociation . In the presence of increasing concentrations of BFA, the GTPγS-induced increase in dissociation rate from the Asn318 mutant GnRH receptor was reversed ($p<0.05$, Figure 4.10e). The pretreatment of COS 7 cells with 100 μM BFA restored the rate for the dissociation of [^{125}I]buserelin from the Asn318 mutant receptor to 85 ± 8% of the control rate. The increased rate of [^{125}I]buserelin dissociation from the wtGnRH receptor caused by GTPγS was not significantly changed by BFA, as shown in Figure 4.10e.

Activation of PLD by the wild type and a mutated form of the 5-HT_{2A} receptor

A wild type and mutant form of the 5-HT_{2A} receptor were used as controls for the effect of mutation at position 318 in the GnRH receptor, to assess the role the N/$DPX_{2-3}Y$ sequence has in receptor coupling to PLD. As with the majority of class I GPCRs, the 5-HT_{2A} receptor contains an $NPX_{2-3}Y$ sequence, and the mutant form of the receptor created by Sealfon *et al* (Sealfon, S. C., et al. 1995), has the conserved asparagine at position 376 replaced by an aspartate residue, thereby producing the $DPX_{2-3}Y$ sequence present in the wild type GnRH receptor. The cDNAs encoding the wt and Asp376 5-HT_{2A} receptor were transiently transfected into COS 7 cells, and the activation of PLD in response to receptor stimulation with increasing concentrations of 5-HT is shown in Figure 4.11. PLD was stimulated potently by the wt and Asp376 mutant 5-HT_{2A} receptor with respective EC_{50} values of 26 ± 13 and 28 ± 13 nM. The maximum level of [^{3}H]PtdBut production by stimulation of the wt and Asp376 5-HT_{2A} receptors was 1.60 ± 0.09 and 3.70 ± 0.70 fold of basal control. There was some evidence to suggest raised basal [^{3}H]PtdBut production in cells expressing the wt but notAsp376 5-HT_{2A} receptor, which may account for the reduced signal in those cells (data not shown). This possibility was not further investigated in the present study. The effects of BFA on the stimulation of PLD by the two 5-HT_{2A} receptors, was consistent with the results obtained using the wt and mutant GnRH receptors. The wt 5-HT_{2A} receptor was sensitive to BFA with significant inhibition of the PLD response, and an IC_{50} for BFA of 47 ± 11 μM. Furthermore the Asp376 mutant was insensitive to the ARF inhibitor with only 24 ± 13% inhibition at 200 μM BFA (Figure 4.12).

4.3 DISCUSSION

Basic receptor signalling

The stimulation of PLD and PLC by the wt and Asn318 mutant GnRH receptor highlighted a radical difference in signal transduction capabilities of the two receptors, and the importance of the conserved $N/DPX_{2-3}Y$ motif. GnRH stimulates a rapid and prominent increase in phosphoinositide turnover in cultured pituitary and αT3-1 gonadotroph cells (Andrews, W. V. and P. M. Conn 1986, Naor, Z. and K. J. Catt 1981, Schrey, M. P. 1985, Snyder, G. D. and J. E. Bleasdale 1982). The stimulation of PLC by the wtGnRH receptor is mediated by $G_{q/11}$ proteins in cultured pituitary cells and in αT3-1 gonadotrophs (Andrews, W. V., et al. 1986, Hsieh, K.-P. and T. F. J. Martin 1992, Limor, R., et al. 1989, Shah, B. H. and G. Milligan 1994). The wt and Asn318 mutant GnRH receptor stimulated InsP production linearly over 40 min, as shown in Figure 4.1. This in agreement with the PLC responses observed after activation of the wt and mutant GnRH receptors expressed in COS 1 and GH_3 cells (Awara, W. M., et al. 1996, Zhou, W. C., et al. 1994). and the non-desensitising InsP production, stimulated by the wtGnRH receptor, reported by Davidson *et al* and Sealfon *et al* (Davidson, J. S., et al. 1994, Sealfon, S. C., et al. 1992). The stimulation of phosphoinositide breakdown by the Asn318 and Asp87-Asn318 mutant GnRH receptors was greatly reduced, compared to the response elicited by the wtGnRH receptor. The poorer coupling to InsP turnover is in agreement with the investigation of the mutant GnRH receptors expressed in COS 1, COS 7 and GH_3 cells (Arora, K. K., et al. 1996, Awara, W. M., et al. 1996, Zhou, W. C., et al. 1994). The reduced

stimulation of InsP production appears to be due to a poorer coupling of the Asn318 and Asp87-Asn318 mutant GnRH receptors to PLC, not just reduced expression of the mutants. Awara *et al*, reported a weak stimulation of phosphoinositide hydrolysis, despite a higher expression of mutant receptors per cell compared to wild type GnRH receptors (Awara, W. M., et al. 1996). Moreover taking into account the lower level of Asn318 GnRH receptor expressed on the cell surface, Arora *et al*, also reported a poorer receptor coupling to PLC (Arora, K. K., et al. 1996). Therefore the change in amino acid residues at positions 87 and 318 may diminish GnRH receptor coupling to InsP production, perhaps as a consequence of a change in receptor conformation and the H-bonding network discussed previously.

Receptor structure and desensitization

The demonstration of an increase in [^{3}H]PtdBut production elicited by the wtGnRH receptor is in agreement with reports of PLD activity stimulated by GnRH in ovarian granulosa and αT3-1cells (Liscovitch, M. and A. Amsterdam 1989, Netiv, E., et al. 1991, Zheng, L., et al. 1994). As with the PLC response, PLD activation by the wt GnRH receptor did not rapidly desensitize. The PAF receptor, which contains a $DPX_{2-3}Y$ motif (Honda, Z., et al. 1991, Kunz, D., et al. 1992, Nakamura, M., et al. 1991, Probst, W. C., et al. 1992), stimulates PLD, and the response wanes after 15-20 minutes. However with the removal of the C-terminal tail, the PLD response is prolonged and does not desensitise (Liu, B., et al. 1997). Similarly the removal of the C-terminal tail of the TRH receptor, abolishes the desensitisation of the InsP production observed with the agonist-stimulated wild type TRH receptor (Yu, R. and P. M. Hinkle 1998). Therefore it is logical to suggest that if the GnRH receptor possessed a C-

terminal tail then, it too would display a desensitisation of its second messenger responses.

Indeed InsP production stimulated by the catfish GnRH receptor, which has a C-terminal tail, and a chimaeric receptor composed of the wt rat GnRH receptor with the C-terminal tail of the TRH receptor did rapidly desensitise (Heding, A., et al. 1998). However as shown in Figure 4.2, the Asn318 and Asp87-Asn318 mutant GnRH receptors elicited a rapid increase in PLD activity, that had reached a plateau after 10 min. The rapid increase in PLD activity induced by agonists such as cholecystokinin (CCK), carbachol, thrombin, vasopressin, bombesin and PAF desensitizes after a short period of activation. (Briscoe, C. P., et al. 1994, Liu, B., et al. 1997, Murthy, K. S. and G. M. Makhlouf 1995, Nieto, M., et al. 1994, Plevin, R. and M. J. O. Wakelam 1992). This period can be seconds in the case of the bombesin receptor (Briscoe, C. P., et al. 1994) and up to 20 minutes as observed for the PAF and CCK receptors (Liu, B., et al. 1997, Murthy, K. S. and G. M. Makhlouf 1995). Therefore the replacement of the single aspartate residue at position 318 with asparagine appears to increase the strength of the receptor coupling to PLD but not PLC, and allow the desensitisation of the receptor activation of PLD, as observed for other receptors. Furthermore the replacement of the asparagine at position 87 with aspartate (in the context of asparagine at 318), does not diminish the increase in the receptors ability to stimulate phosphatidylcholine breakdown.

Pathways to PLD via small G proteins or PLC

The Asn318, but not the wild type GnRH receptor activated PLD in an ARF-dependent manner as shown by the results in Figure 4.5. The BFA

sensitivity of the Asn318 GnRH receptor stimulation of PLD, is in agreement with the hypothesis that $NPX_{2-3}Y$-containing receptors couple to PLD via small G proteins such as ARF and Rho. The demonstration of BFA-sensitivity in the activation of PLD by the M_3, B_2 and H_1, but not the thrombin and TXA_2 receptors, described in Chapter 3, implicated the conserved TMD 7 motif in the regulation of G protein coupling. The effect of the substitution of aspartate at position 318 in the wt GnRH receptor with asparagine, strongly supports the idea of a pivotal role for the $N/DPX_{2-3}Y$ sequence in the receptor interaction with G proteins. Furthermore the effect that mutating the $NPX_{2-3}Y$ sequence in the GnRH receptor has on G protein coupling was not a receptor-specific one, as the substitution of the asparagine at position 376 in the human 5-HT_{2A} receptor, with aspartate removed the BFA-sensitivity of the 5-HT-induced activation of PLD (Figure 4.12).

The dramatic influence of the amino acid substitution at the 7.49 position on G protein coupling was further emphasised by the results from experiments using the PLC inhibitor U73122. The activation of PLD by the wtGnRH and Asp376 mutant 5-HT_{2A} receptors, was sensitive to the PLC inhibitor U73122. This further illustrates the apparent sensitivity of $DPX_{2-3}Y$ receptors to U73122 in their activation of PLD, a suggestion arising from the stimulation of PLD by the thrombin receptor, presented in Chapter 3. Furthermore the Asn318 mutant GnRH and wt5-HT_{2A} receptors activate PLD in a U73122-insensitive manner, as with the other $NPX_{2-3}Y$ receptors investigated in Chapter 3.

The modification of the GnRH and 5-HT_{2A} signalling characteristics by a single amino acid change, is one of many examples of a change in receptor activation of G protein signalling pathways, due to point

mutations. Indeed a relationship between naturally occurring point mutant GPCRs, abnormal constitutive activity and disease states has been reported . Mutation of an aspartate residue in the luteinising hormone receptor, conserved within the TMD 6 of glycoprotein hormone receptors is linked with precocious male puberty (Shenker, A., et al. 1993). Furthermore the mutation of Lys296 and Glu113 in the TMDs 7 and 3 respectively of rhodopsin, is linked with retinitis pigmentosa (Robinson, P. R., et al. 1992). The mutation of single amino acid residues within the carboxy terminus of the i3, a domain considered to be directly involved in G protein coupling, causes constitutive activation of the α_{1B}, α_{2C10} and β_2-adrenergic receptors and activation of their respective G_q, G_i and G_s coupling (Kjelsberg, M. A., et al. 1992, Ren, Q., et al. 1993, Samama, p., et al. 1993). The modulation of one second messenger pathway and not another by a single amino acid substitution, as seen for the differing PLC and PLD responses of the wt and Asn318 mutant GnRH receptor is also observed. The mutation of an alanine residue in i3 of the thyrotropin (TSH) receptor caused the loss of the phosphoinositide hydrolysis, but not the cAMP response (Kosugi, S., et al. 1992). Also of interest was that the mutation of an equivalent alanine residue in the α_{1B}-adrenergic receptor increased the potency of agonists for stimulating PLC activity by 2 orders of magnitude (Cotecchia, S., et al. 1990).

Interhelical interactions in GPCRs

Therefore the pervasive influence of a single amino acid change on receptor activity and G protein coupling is a well reported phenomena. However the consequence of changes to the conserved Asn or Asp in the $N/DPX_{2-3}Y$ motif has not been detailed currently. Work reported by several groups points to a close interaction of the asparagine in TMD 2

and the aspartate in TMD 7, in the GnRH receptor (Arora, K. K., et al. 1996, Awara, W. M., et al. 1996, Zhou, W. C., et al. 1994). The replacement of Asn87 in TMD 2 abolished the ligand binding properties and related second messenger responses of the GnRH receptor (Cook, J. V., et al. 1993, Zhou, W. C., et al. 1994). Introducing a second mutation in TMD 7, changing Asp318 to Asn (which recreates the pattern seen in other GPCRs) led to restoration of a receptor with high affinity agonist and antagonist binding (Awara, W. M., et al. 1996, Mitchell, R., et al. 1998, Zhou, W. C., et al. 1994). The double mutant also coupled to InsP production, although the stimulation of phosphoinositide hydrolysis was weaker than that seen with the wt GnRH receptor (Awara, W. M., et al. 1996, Zhou, W. C., et al. 1994). This is in agreement with the results presented in Figure 4.1. Moreover this reduction in G protein coupling does not appear to be a result of the poor expression of mutant receptor on the cell surface. Even after taking into account the lower number of mutant GnRH receptors, the hydrolysis of phosphatidylinositol 4,5 bisphosphate, was poorly stimulated by the Asn318 and Asp87-Asn318 GnRH receptors (Arora, K. K., et al. 1996, Awara, W. M., et al. 1996). The restoration of binding and G protein coupling by a reciprocal mutation supports the proximity of TMD 2 and 7, and the specific interaction of the conserved polar residues in either helix.

The introduction of the Asp at position 87 in TMD 2 is proposed to disrupt the H bonding interactions of the GnRH receptor. Aspartate is assumed to be ionised physiologically and act as an H-bond acceptor through its side chain. In the Asp87 GnRH receptor, the Asp residues at position 87 and 318 would electrostatically repel each other, thereby affecting receptor structure. Asparagine can act as both a hydrogen-bond

acceptor and donor through its side chain. Therefore the introduction of the Asn residue in the Asp87-Asn318 double mutant GnRH receptor, would allow these two loci to H-bond, and participate again in the putative network of polar interactions described previously (Fanelli, F., et al. 1995, Konvicka, K., et al. 1998, Oliveira, L., et al. 1994, Perlman, J. H., et al. 1997, Scheer, A., et al. 1996, Scheer, A., et al. 1997). However in the Asp376 mutant of the 5-HT_{2A} receptor, where there is an Asp in both TMD 2 and TMD 7, InsP production was still stimulated by the receptor. An explanation for the difference observed between the GnRH and 5-HT_{2A} receptor mutants, was proposed by Sealfon *et al*, who suggest that the Asp residue, introduced into TMD 7 may be protonated and therefore act as a hydrogen donor, like the Asn residue, normally present (Sealfon, S. C., et al. 1995). A similar explanation for the retention of receptor stimulation of PLC by a mutant TRH receptor, with Asp residues in TMDs 2 and 7 was reported recently (Perlman, J. H., et al. 1997). In the wt 5-HT_{2A} receptor the conserved Asn at position 1.50 is proposed to H-bond with Asp 2.50 and Asn 7.49 (Konvicka, K., et al. 1998). In the mutant 5-HT_{2A} receptor, there may still exist H-bond interactions between the Asp residues at positions 2.50 and 7.49 and the conserved Asn 1.50. This polar bonding between helices may counteract the hypothetical electrostatic incompatibility and allow some level of normal G protein coupling. A similar explanation may be applied to the functionally competent mutant TRH receptor, which also has Asp residues at position 2.50 and 7.49 and is proposed to have H-bonding between positions 1.50, 2.50 and 7.49 (Perlman, J. H., et al. 1997). The local micro-enviroment of each receptor will determine the importance of either an Asp or Asn in the transmembrane α-helices 2 and 7. However the receptor coupling to G

proteins and second messenger responses in all cases reported was strongest in the wild type/evolutionarily conserved structures.

There was an enhancement of the stimulation of PLD by the Asn318 and Asp87-Asn318 mutant GnRH receptors, despite a poor receptor coupling to InsP production and reduced receptor number (Figure 4.2). This further implicates the $N/DPX_{2-3}Y$ motif in the control of G protein coupling. Specifically, changes in the motif of the GnRH receptor generate an increased competency of receptor coupling to PLD. Moreover the mutant but not wild type receptors coupled to a BFA-sensitive pathway in the activation of PLD and could associate closely with ARF and Rho proteins. Therefore the presence of the NPLIY motif in TMD 7 would appear to be necessary for GPCR coupling to these small G proteins in the stimulation of PLD. The capacity of the Asn at position 318 to be an H-bond donor, may be the important determinant in the mutant GnRH receptor, and other GPCRs, that controls the coupling to PLD. Investigation of the conserved aspartate in TMD 2 and asparagine in TMD 7, of the 5-HT_{2A} receptor by Sealfon *et al*, implicated an H-bond interaction between these amino acid residues (Sealfon, S. C., et al. 1995). A mutant of the 5-HT_{2A} receptor, with the Asn at position 376 replaced with an Asp residue, was able to stimulate InsP production (Sealfon, S. C., et al. 1995). Furthermore the coupling of this Asp376 5-HT_{2A} receptor to PLC was only slightly reduced compared to that seen with the wt 5-HT_{2A} receptor. However the importance of the $NPX_{2-3}Y$ motif in GPCR coupling to PLD, was underscored by the loss of the involvement of ARF in the activation of PLD by the mutant Asp376 5-HT_{2A} receptor (Figure 4.12).

Receptor selective interaction with G proteins

The close association of the M_3 receptor with the small G proteins ARF1/3 and RhoA reported in Chapter 3 and by Mitchell *et al* (Mitchell, R., et al. 1998), was reproduced with the Asn318 mutant but not the wt GnRH receptor. Specific binding of the stable GnRH analogue, [^{125}I]buserelin to immunoprecipitates prepared using polyclonal antibodies to ARF1/3 and RhoA, and COS 7 cells expressing the Asn318 GnRH receptor was not observed following the transient expression of the wt GnRH receptor. This further supports the idea that the selectivity of receptor coupling to ARF and RhoA is based on the presence of the $NPX_{2\text{-}3}Y$ and not the alternative $DPX_{2\text{-}3}Y$ sequence. As observed for the association of the M_3 receptor with ARF and Rho proteins (Figure 3.8), there is a prerequisite for exposure of the GnRH receptor to agonist, which may be required in order to induce the translocation of the G proteins to the cell membranes as reported previously (Houle, M. G., et al. 1995, Malcolm, K. C., et al. 1996, Rümenapp, U., et al. 1995), where they may then interact with receptor. Furthermore the rate of buserelin dissociation from the wt and Asn318 GnRH receptor was modulated in a fashion that further suggested the selective coupling of the receptors to trimeric and monomeric G proteins respectively (Figure 4.10).

The activation of a broad spectrum of G proteins by GTPγS or GPPNHP decreased the wt and Asn318 GnRH receptor's affinity for buserelin, thus increasing the rate of ligand dissociation from the receptors (Figure 4.10a and b). Incubation of digitonin-permeabilized COS 7 cells expressing the wt or Asn318 mutant GnRH receptor with $GPPCH_2P$ decreased the half-life for buserelin dissociation from the Asn318 but not the wt GnRH receptor. The work described in Chapter 3 on the modulation of M_3 receptor affinity for agonist and activation of PLD points to the selective

activation of small G proteins by βγ-methylene analogues of GTP. This is in agreement with the reported potency and selectivity of these analogues for monomeric G proteins such as rab5, ARF and others (Gill, D. M. and J. Coburn 1987, Hoffenberg, S., et al. 1996). Therefore the $NPX_{2-3}Y$ and not the $DPX_{2-3}Y$ containing GnRH receptor appears to interact specifically with small G proteins and this is consistent with the association of the M_3 receptor and these G proteins reported in Chapter 3. Furthermore the inferred selective stimulation of trimeric G proteins with BeF_3^- (Bigay, J., et al. 1987, Kahn, R. A. 1991), reduced only the wt GnRH receptor's half-life for [^{125}I]buserelin dissociation. This is supportive of the hypothesis of significant coupling of trimeric G proteins to the wt and not the Asn318 mutant GnRH receptor after agonist exposure. These results would appear to confirm again that the two forms of the GnRH receptor couple at least in part to two populations of G proteins, in a manner reminiscent of the unprimed and primed M_3 receptor.

Conformational changes in receptor activation

The participation of the conserved Asn/Asp in TMD 7 and the conserved Asp/Asn in TMD 2 in the regulation of a H-bonding network has been discussed previously in Chapter 3. The results presented in Chapter 3 and in this Chapter suggest that changes in one locus of this network have dramatic effects on the receptor-G protein coupling behaviour. In the GnRH, 5-HT_{2A}, TRH, M_3 and α_{1B} receptors, mutagenesis and computer molecular modelling have all suggested the conformational change leading to the receptor coupling to G proteins was regulated by either all or a selection of the polar network of amino acids; N1.50, D/N2.50, R3.50, N/D7.49 and Y7.53 (Ballesteros, J., et al. 1998, Fanelli, F., et al. 1995, Luo, X., et al. 1994, Perlman, J. H., et al. 1997, Scheer, A., et al. 1996, Scheer, A., et al.

1997, Sealfon, S. C., et al. 1995, Zhou, W. C., et al. 1994). The polar network, as described in Chapter 3 undergoes a conformational change upon ligand binding, which is suggested to lead to the increased accessibility of the putative G protein coupling domains with the receptor (Fanelli, F., et al. 1995, Konvicka, K., et al. 1998, Oliveira, L., et al. 1994, Scheer, A., et al. 1996, Scheer, A., et al. 1997). Computer simulations of the wt and a mutant Asn120 5-HT_{2A} receptor indicate that TMDs 5 and 6 are the foci for receptor conformational changes, initiated with TMD 7 (Luo, X., et al. 1994, Sealfon, S. C., et al. 1995, Zhang, D. and H. Weinstein 1993). Since the i3 loop which links these two α-helices is considered to be a major G protein coupling domain in GPCRs, the movement of TMDs 5 and 6 is likely to greatly influence the receptor stimulation of second messengers. A series of site-directed spin labelling studies using the rhodopsin receptor, has implicated a significant movement away from each other of TMDs 3 and 6 and a movement of i3 upon photoactivation (Altenbach, C., et al. 1996, Farahbakhsh, Z. T., et al. 1993, Farahbakhsh, Z. T., et al. 1995, Farrens, D. L., et al. 1996). The TMD 6 is proposed to twist by 30° on its axis and to lean outwards away from the central pocket formed by the other TMDs (Altenbach, C., et al. 1996, Farahbakhsh, Z. T., et al. 1993, Farahbakhsh, Z. T., et al. 1995, Farrens, D. L., et al. 1996). Similarly the i3 loop of the yeast α–factor pheromone receptor is given greater accessibility upon agonist binding, as shown by the greater proteolytic cleavage the i3 loop (Bukusoglu, G. and D. D. Jenness 1996).

The $N/DPX_{2\text{-}3}Y$ sequence was suggested to be ideally placed to receive the signals of ligand binding to a receptor, and transduce them to the G protein coupling domains of GPCRs (Donnelly, D., et al. 1994, Findlay, J. B. C., et al. 1993). The results reported here and elsewhere indicate that

the N/DPX$_{2-3}$Y sequence (and specifically the identity of the polar amino acid at position 7.49 in GPCRs) is a major determinant in the control of G protein coupling. Through the interaction of the conserved Asn or Asp residues at positions 7.49 and 2.50 with the other polar amino acids forming the network of H-bonds (most crucially the conserved Asn at position 1.50), the functionally competent conformation of receptors appears to be maintained. One important effect of the N/DPX$_{2-3}$Y motif is the regulation of receptor coupling to ARF and Rho proteins in the receptor-mediated activation of PLD by agonist. The ability of asparagine as opposed to aspartate, to act as both hydrogen bond donor and acceptor, may be the reason behind the difference in receptor activation of PLD. The negative charge associated with the aspartate in TMD 7 may also play a role in the local control of receptor conformation. Whatever the influence of the different amino acid composition of the highly conserved motif, now along with the second and third intracellular loops, the N/DPX$_{2-3}$Y motif can be seen as a central switch in the control of class I, GPCR signal transduction.

Figure 4.1

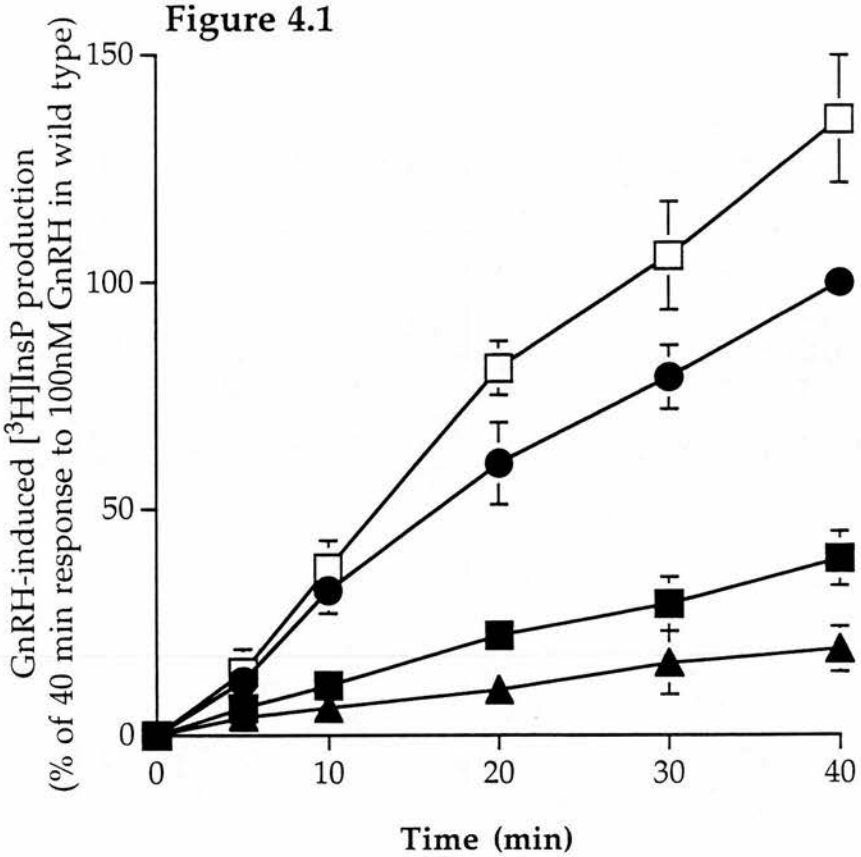

GnRH-induced [3H]InsP production
(% of 40 min response to 100nM GnRH in wild type)
150
100
50
0
0
10
20
30
40
Time (min)

Figure 4.2

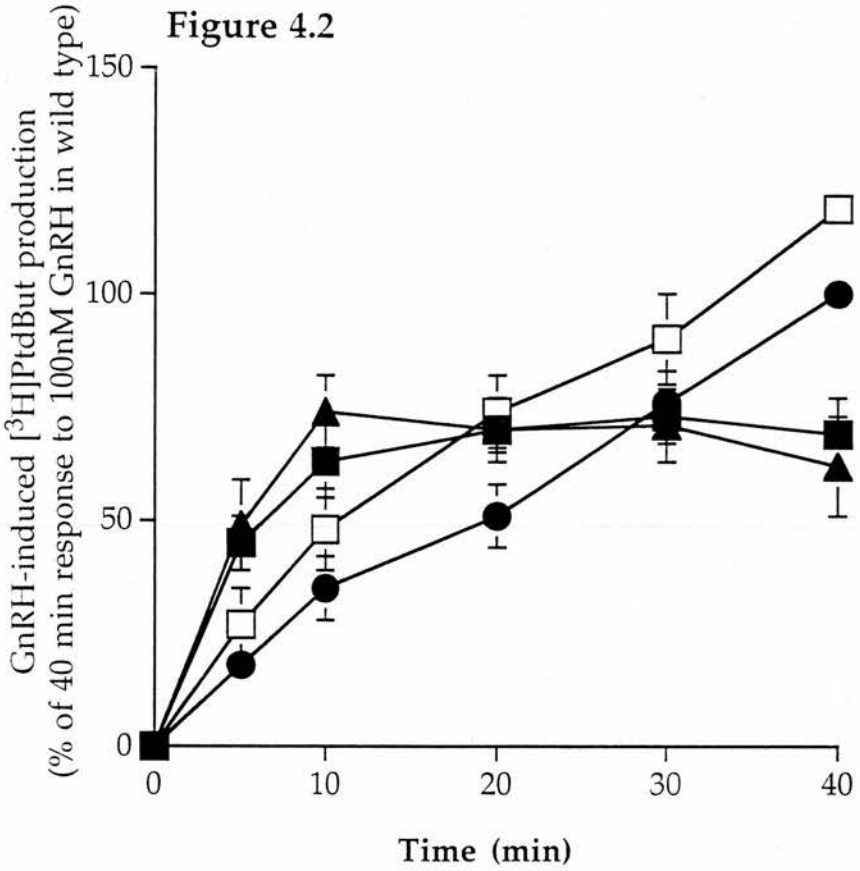

GnRH-induced [3H]PtdBut production
(% of 40 min response to 100nM GnRH in wild type)
150
100
50
0
0
10
20
30
40
Time (min)

Figure 4.3

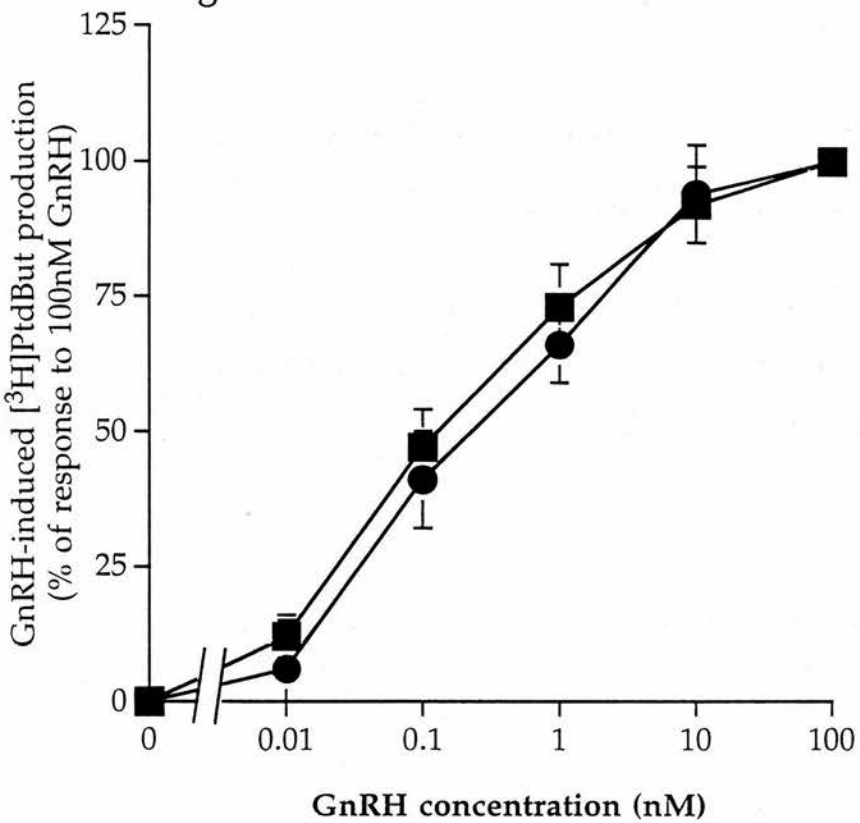

125
100
75
50
25
0
GnRH-induced [3H]PtdBut production
(% of response to 100nM GnRH)
0
0.01
0.1
1
10
100
GnRH concentration (nM)

Figure 4.4

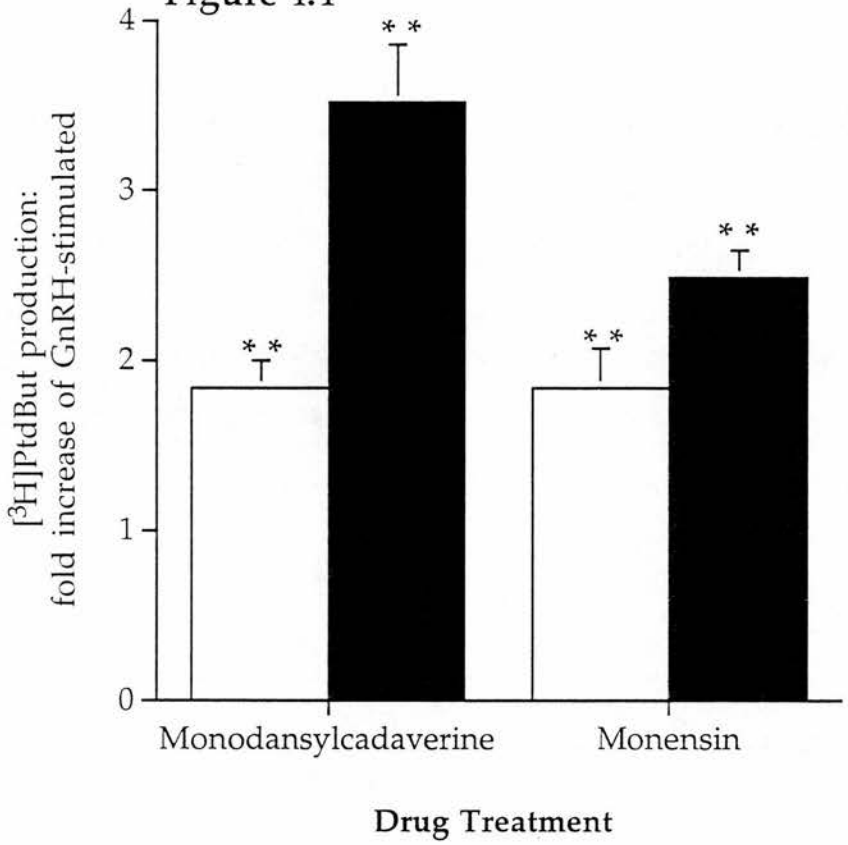

[3H]PtdBut production:
fold increase of GnRH-stimulated
4
3
2
1
0
* *
* *
* *
* *
Monodansylcadaverine
Monensin
Drug Treatment

Figure 4.5

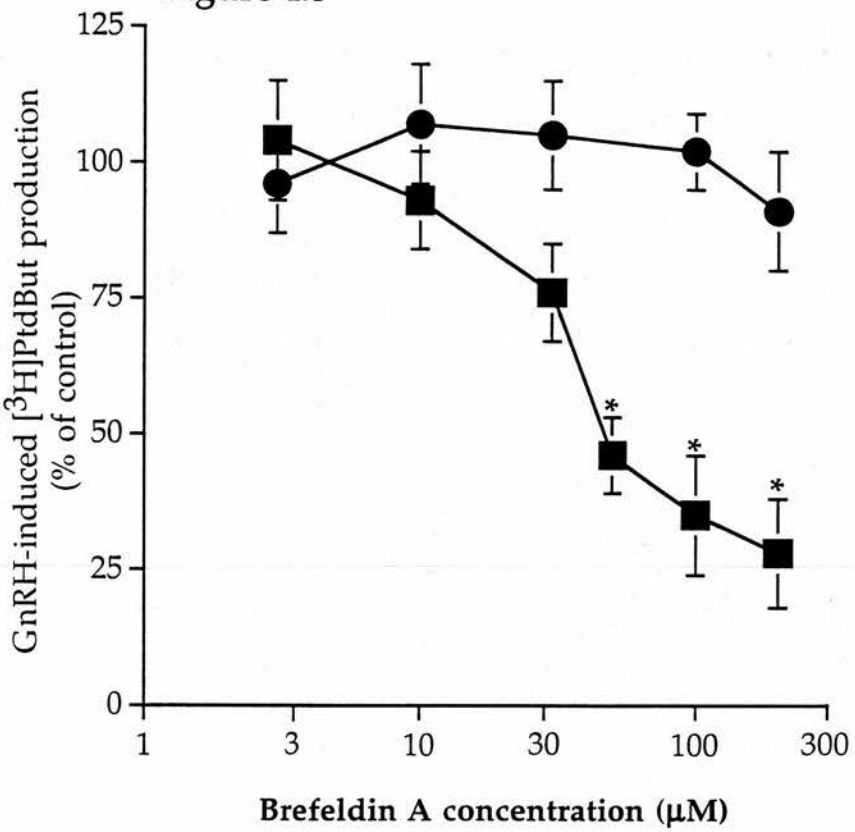

125
100
75
50
25
0
1
3
10
30
100
300
GnRH-induced [3H]PtdBut production
(% of control)
Brefeldin A concentration (μM)
*
*
*

Figure 4.6

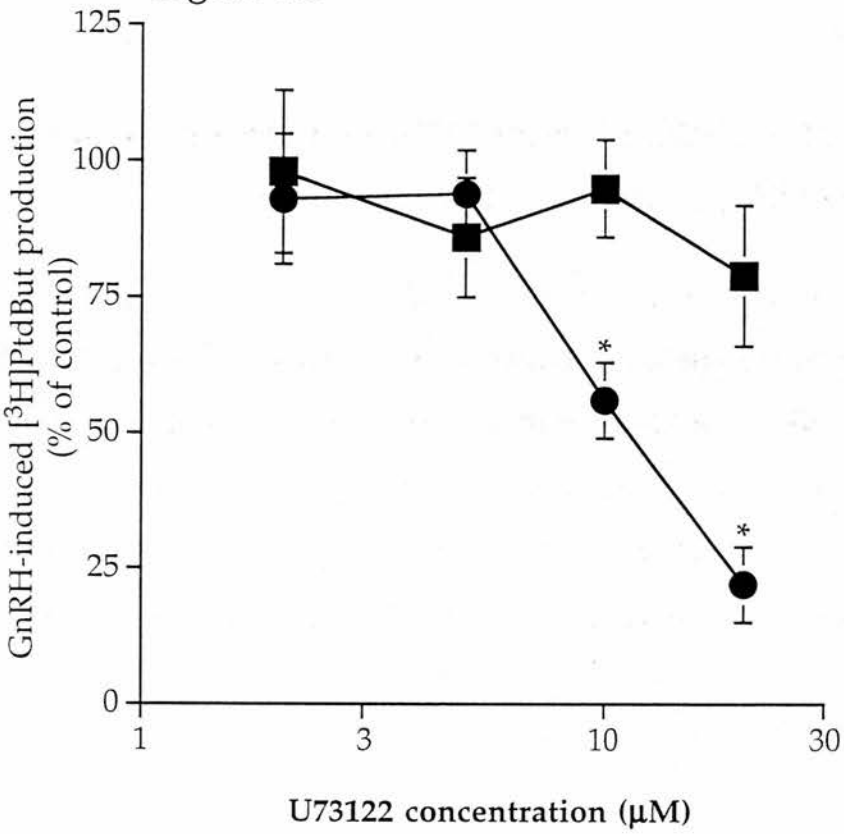

125
100
75
50
25
0
GnRH-induced [3H]PtdBut production
(% of control)
1
3
10
30
*
*
U73122 concentration (μM)

Figure 4.7

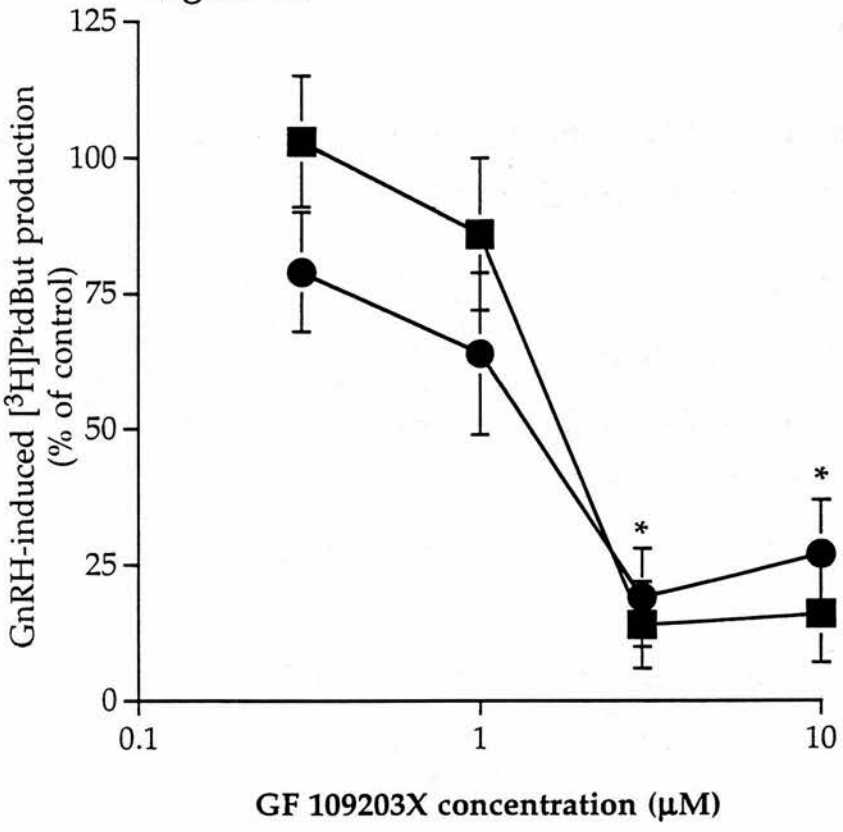

125
100
75
50
25
0
0.1
1
10
*
*
GnRH-induced [3H]PtdBut production
(% of control)
GF 109203X concentration (μM)

Figure 4.8

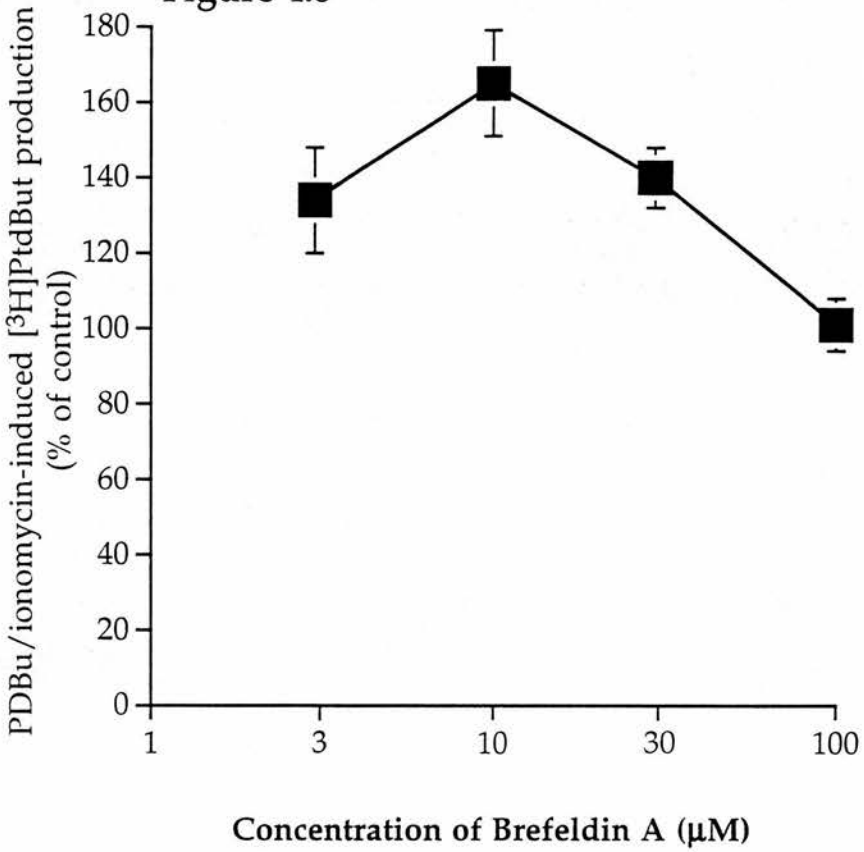

PDBu/ionomycin-induced [3H]PtdBut production
(% of control)
180
160
140
120
100
80
60
40
20
0
1
3
10
30
100
Concentration of Brefeldin A (μM)

Figure 4.9

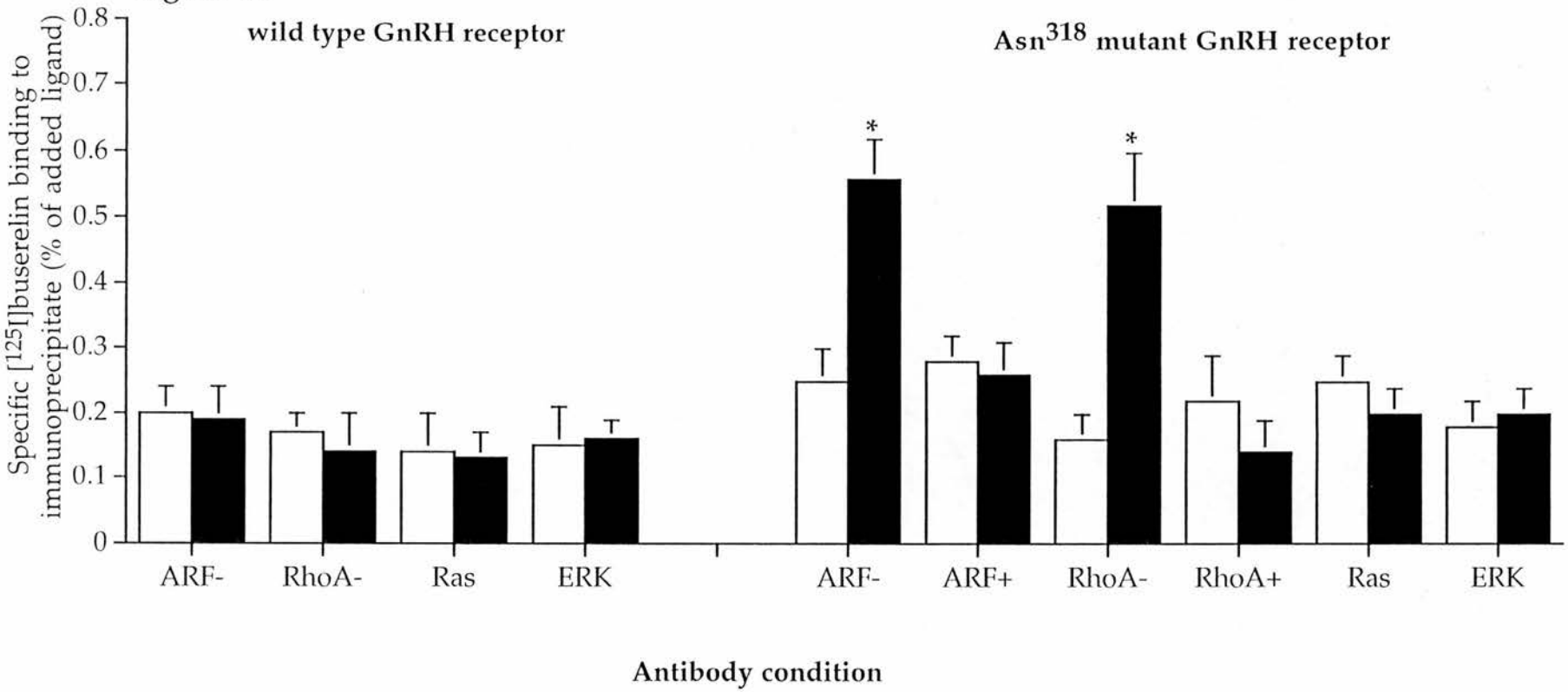

wild type GnRH receptor
Asn318 mutant GnRH receptor
Specific [125I]buserelin binding to immunoprecipitate (% of added ligand)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
ARF-
RhoA-
Ras
ERK
ARF-
ARF+
RhoA-
RhoA+
Ras
ERK
Antibody condition

Figure 4.10

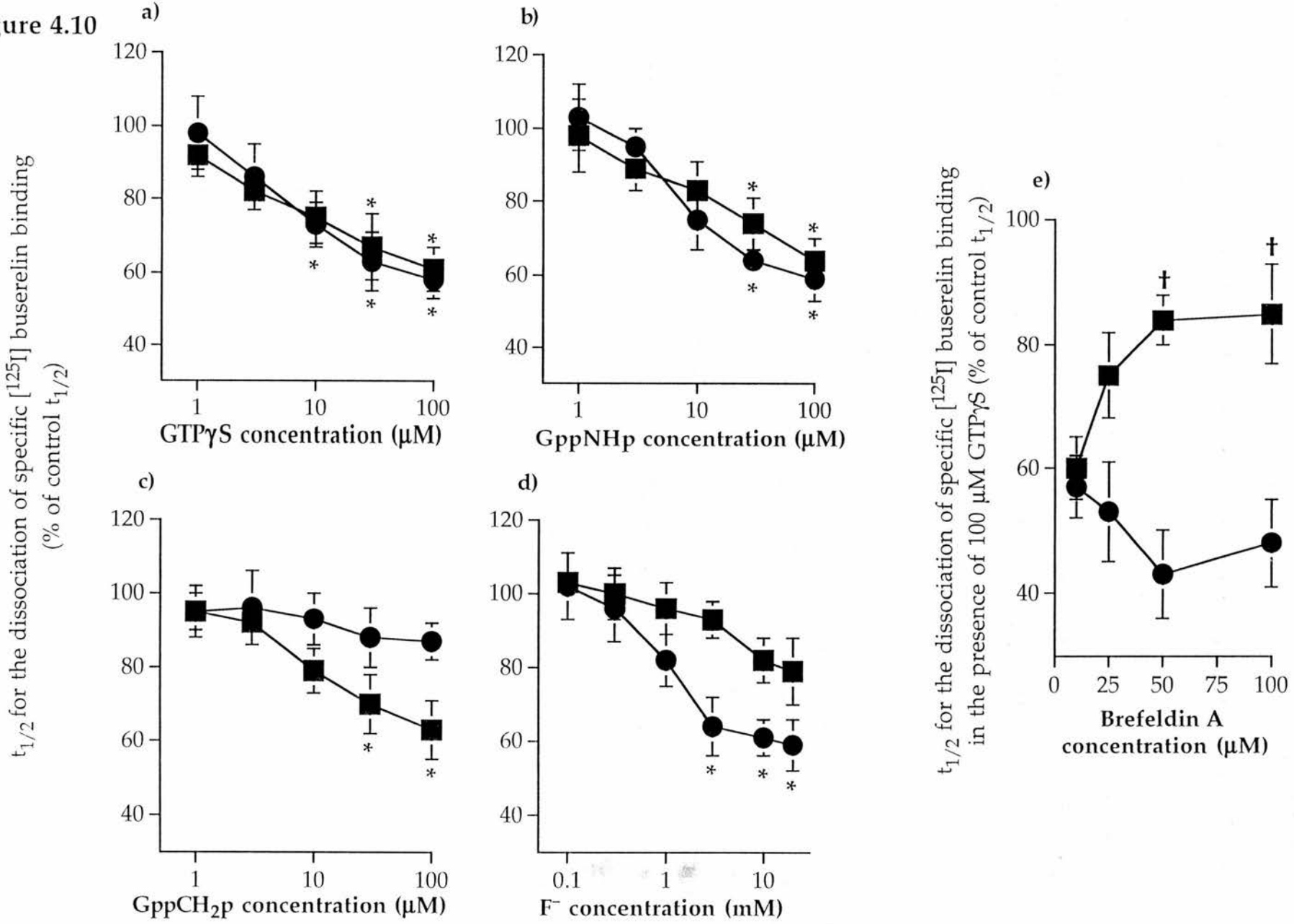
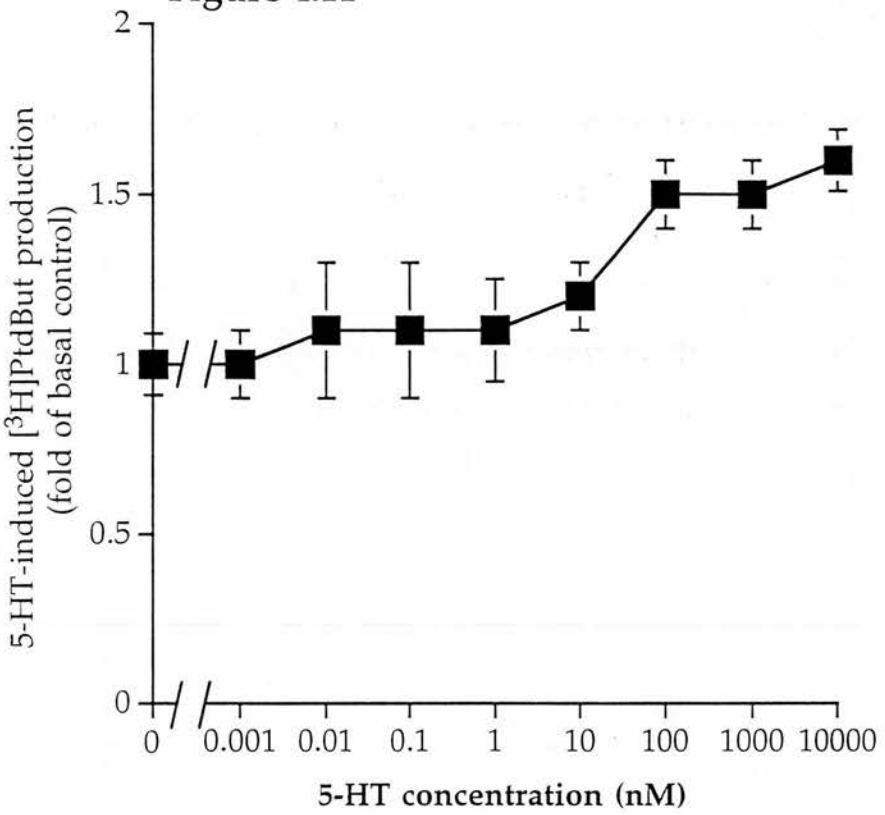

a)
b)
c)
d)
e)
$t_{1/2}$ for the dissociation of specific [^{125}I] buserelin binding (% of control $t_{1/2}$)
GTPγS concentration (μM)
GppNHp concentration (μM)
GppCH$_2$p concentration (μM)
F$^-$ concentration (mM)
$t_{1/2}$ for the dissociation of specific [^{125}I] buserelin binding in the presence of 100 μM GTPγS (% of control $t_{1/2}$)
Brefeldin A concentration (μM)

Figure 4.11


5-HT-induced [3H]PtdBut production
(fold of basal control)
2
1.5
1
0.5
0
0
0.001
0.01
0.1
1
10
100
1000
10000
5-HT concentration (nM)

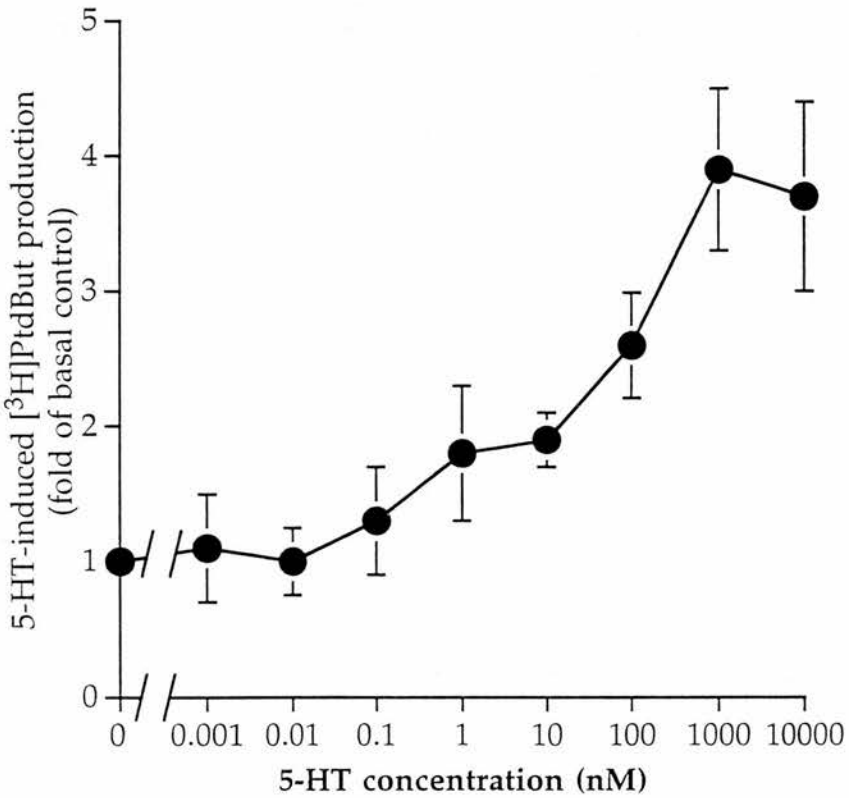
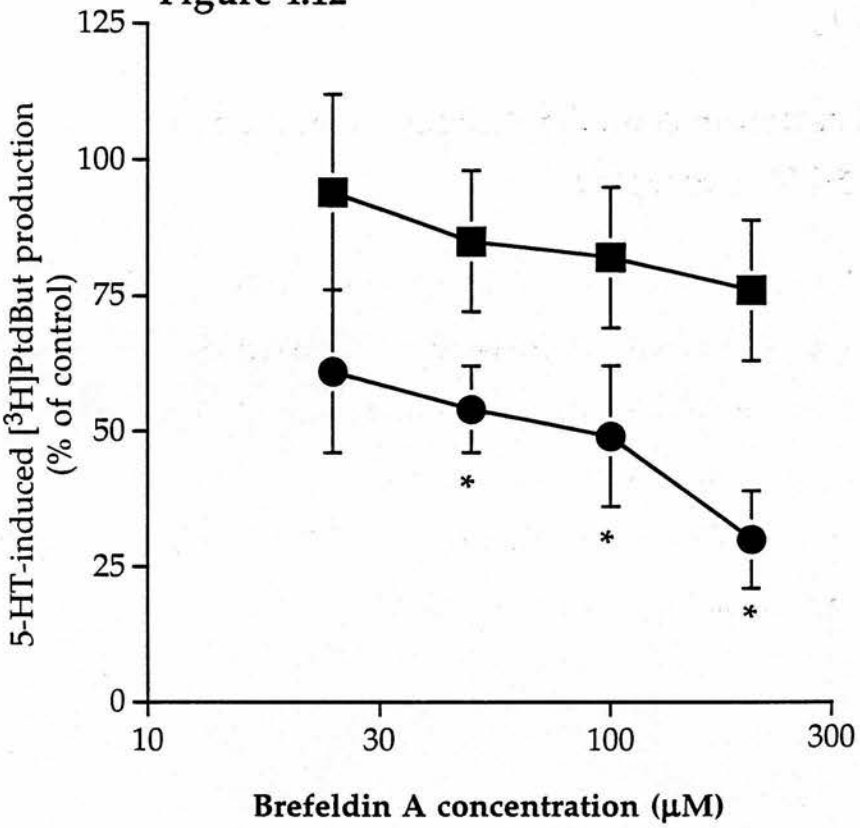

5-HT-induced [3H]PtdBut production
(fold of basal control)
5
4
3
2
1
0
0
0.001
0.01
0.1
1
10
100
1000
10000
5-HT concentration (nM)

Figure 4.12


125
100
75
50
25
0
5-HT-induced [3H]PtdBut production
(% of control)
10
30
100
300
Brefeldin A concentration (μM)
*
*
*

CHAPTER 5

Activation of PLD by VIP/PACAP/secretin family receptors

5.1 INTRODUCTION

Recently, a family of GPCRs was recognised as being structurally distinct from the rhodopsin (class I) family. Known as the secretin/glucagon (class II) receptor family, this group shares few of the specific sequence motifs of the rhodopsin receptor family and has less than 12% sequence homology with its members, but still possesses the same predicted molecular architecture of seven transmembrane helices and the ability to transduce extracellular stimuli via an analogous sequence of molecular events (Segre, G. V. and S. R. Goldring 1993). Receptors within the secretin/glucagon family show between 30% and 51% homology with each other and share characteristics such as the conservation of 6 specific cysteine residues within the N-terminal extracellular domain, activation of adenylate cyclase by coupling to the heterotrimeric G protein G_s, multiple consensus N-glycosylation sites, a large N-terminal extracellular domain (>120 amino acids) and multi-intron genes (Arimura, A. and S. Shioda 1995, Laburthe, M. and A. Couvineau 1988, Laburthe, M., et al. 1996, Segre, G. V. and S. R. Goldring 1993). Recently two distinct receptors for Vasoactive Intestinal Polypeptide (VIP) have been cloned. In 1992, Ishihara *et al.* cloned the VIP_1 receptor using a rat lung cDNA library and the VIP_2 receptor was cloned soon after from rat pituitary (Lutz, E. M., et al. 1993). Both receptors can potently activate adenylate cyclase via coupling to G_s and raise intracellular cAMP levels (Ishihara, T., et al. 1992, Kermode, J. C., et al. 1992, Lutz, E. M., et al. 1993, Usdin, T. B., et al. 1994). The coupling of VIP receptors to inositol phosphate (InsP) metabolism is not so well defined, although VIP was shown to increase the breakdown of inositol phosphates in rat astroglia (Fatatis, A., et al. 1994), and the mouse homologue of the VIP_2 receptor stimulated calcium-activated

chloride currents, which is suggestive of a coupling to the PLC pathway (Inagaki, N., et al. 1994). Also agonist can mobilise calcium from intracellular calcium stores in cells expressing the human VIP_1 receptor, which strongly suggests the involvement of phosphoinositide breakdown (Sreedharan, S. P., et al. 1994). Mackenzie *et al.* have shown that the rat VIP_1 and VIP_2 receptors when expressed transiently in COS-7 cells can couple to PLC in a pertussis toxin-sensitive manner, suggesting the involvement of a member of the G_i family of heterotrimeric G proteins (MacKenzie, C. J., et al. 1996). Moreover the rat VIP_1 receptor stably expressed in chinese hamster ovary (CHO) cells has also been shown to stimulate phospholipase C (PLC) in a pertussis toxin-sensitive manner (van Rampelbergh, J., et al. 1997). Whilst the activation of PLD is observed in response to a wide range of physiological stimuli, any ability of the rat VIP_1 and VIP_2 receptors to activate PLD has never been explored. This question was investigated here with attention paid to the potential role of small G proteins such as ARF in this activation. The member of the secretin/calcitonin family of receptors most closely related to the VIP_1 and VIP_2 receptors is the PACAP receptor, with 51% similarity (Ishihara, T., et al. 1992). The respective VIP and PACAP peptides ligands are also the most alike within this peptide family, having 70% identity (Arimura, A. 1992, Miyata, A., et al. 1990).

Independently of each other, 6 labs reported cloning of the receptor for PACAP (Hashimoto, H., et al. 1993, Hosoya, M., et al. 1993, Morrow, J. A., et al. 1993, Pisegna, J. R. and S. A. Wank 1993, Spengler, D., et al. 1993, Svoboda, M., et al. 1993). The PACAP receptor was found to have 6 splice variants which all coupled to G_s, as with all other members of the secretin receptor family (Laburthe, M., et al. 1996, Segre, G. V. and S. R. Goldring

1993, Spengler, D., et al. 1993), but differed in the structure of the third intracellular loop (i3). The receptors can exist in a short form or in one of 5 longer splice variants characterised by insertion into i3 of 28 amino acid cassettes termed "hip", "hop-1" and "hop-2", either alone or in combination . The short form of the receptor or splice variants containing the hop-1, hop-2 have an almost identical potency (≈ 0.4 nM) for the PACAP-38-evoked activation of adenylate cyclase in LLC-PK1 cells. However the PACAP-hip receptor was 15 times less potent at increasing cAMP levels, while the PACAP-hip-hop1/2 splice variants have an intermediate phenotype (Journot, L., et al. 1995, Spengler, D., et al. 1993). The potency of PACAP-38 also differed in its stimulation of InsP breakdown through the PACAP receptor splice variants. The PACAPshort, hop-1 and hop-2 forms of the receptor stimulated InsP hydrolysis with equal potency (as with the cAMP response), the PACAP-hip receptor did not induce any detectable phosphoinositide breakdown in LLC-PK1 cells, once again the PACAP-hip-hop1/2 displayed an intermediate phenotype (Journot, L., et al. 1995, Spengler, D., et al. 1993). A similar profile for the stimulation of calcium currents in *Xenopus* oocytes was observed in oocytes micro-injected with the 6 different receptor mRNAs. Once again the PACAP-hip receptor did not induce a calcium current (Journot, L., et al. 1995, Spengler, D., et al. 1993). The hip cassette may represent a non-functional insert that interferes with the productive coupling of the PACAP receptor to G proteins. The ability of the $PACAP_{short}$ form (containing no hip or hop cassettes) and $PACAP_{long}$ form receptor (including hop-1) to activate PLD in response to agonist is described here and the role of ARF in this activation was also addressed. Chimaeric receptor constructs were used to address what effect the different i3 structure of the PACAP receptor splice variants would have

on their ability to activate PLD. The chimaeric receptor constructs comprised the VIP_2 receptor with its i3 replaced by that of the $PACAP_{short}$ or $PACAP_{long}$ receptor.

5.2 SPECIFIC METHODOLOGY

Construction of chimaeric receptors

Chimeric receptors were made by Dr Eve Lutz in our laboratory by exchanging the i3 domain of the rat VIP_2 receptor and either the short (missing amino acids 348-376 in the third intracellular loop) or the long form of the rat PACAP receptor, at exchange sites on TMD 5 and TMD 7 (Fig. 5.9). This was achieved using cDNAs encoding the rat VIP_2 receptor (R4, pBluescript) and the short (R7b, pBluescript) and long (R7/9.1, pBluescript) forms of the rat PACAP receptor. The first domain exchange was made by utilising a conserved restriction (*Hinc*II) site in the region of the cDNAs encoding the 5th transmembrane region of the VIP_2 and PACAP receptors. After digestion with *Hinc*II, the appropriate cDNA fragments were gel purified then ligated with T_4DNA ligase (Promega). These were inserted into pBluescript for selection of appropriate clones by sequence analysis of the domain exchange region. The second domain exchange within TMD 7 was made by overlap extension polymerase chain reaction (PCR) mutagenesis, described previously (Huang, Z., et al. 1995). For each cDNA a set of four oligonucleotide primers were used, two external primers derived from flanking sequences of pBluescript and two internal primers one of which (the overlap primer) contained sequences derived from both receptor encoding cDNAs which spanned the TMD 7 junction site. The cDNA specific primer contained sequences

complementary to the 5′ end of the overlap primer, corresponding to the cDNA encoding the receptor portion 5′ of the junction site. In the first round of PCR amplification the 5′ region encoding the N-terminal end of the chimaeric receptor was amplified with the cDNA specific primer along with the corresponding flanking external primer while the 3′ region encoding the C-terminal end was amplified with the overlap primer and corresponding flanking external primer. PCR reactions were set up in 100 µl volumes containing 15 ng cDNA, 15 pmol of the 5′ and 3′ primer, in the PCR buffer with 2 mM $MgCl_2$, 100 nM dNTPs and 10% DMSO and overlaid with mineral oil. The reaction was heated to 95°C for 5 min, then maintained at 80°C while adding 2.5U Pfu (*Pyrococcus furiosus*) polymerase (Stratagene), after which the reaction was put through 30 cycles with denaturing at 94°C (1 min), annealing at 57°C (1 min) and extension at 72°C (3 min). After the first round of PCR, 10 µl samples were analysed by electrophoresis. The remaining PCR reactions were purified by extracting with the Wizard cDNA purification system (Promega), then in the second round of PCR amplification 1 µl volumes of each appropriate extract were mixed and amplified using the flanking pBluescript primers under the same conditions as the first round of amplification. The polymerase enzyme was removed by the Wizard cDNA purification system and the reaction digested with either *EcoR*I or *EcoR*I/*Xho*I, then run on agarose gels for size selection. These were ligated into pBluescript for selection of appropriate clones by sequence analysis, then inserted into the expression vector pcDNA 1 (InVitrogen, R&D Systems Europe Ltd., Abingdon, UK) for functional expression in COS 7 cells.

5.3 RESULTS

VIP receptor signalling in response to agonist.

The VIP_1 and VIP_2 receptors expressed stably in CHO cells clearly displayed comparable ability to activate phospholipase D in response to VIP. Their responses were concentration-dependent, with EC_{50} values of 6.19 ± 2.48 and 5.34 ± 1.22 nM respectively, as shown in Fig. 5.1. The ability of VIP_1 and VIP_2 receptors to activate adenylate cyclase is well documented (Ishihara, T., et al. 1992, Lutz, E. M., et al. 1993, Usdin, T. B., et al. 1994) and the ability to activate inositol phosphate production has also been shown (MacKenzie, C. J., et al. 1996, Sreedharan, S. P., et al. 1994, van Rampelbergh, J., et al. 1997). The activation of PLD by the glucagon receptor was the first report of a class II receptor coupling to PLD (Pittner, R. A. and J. N. Fain 1991). However the present results are, the first explicit demonstration of the activation of PLD by the VIP and PACAP receptors and a description of the mechanism of that activation. The VIP_1 receptor elicits a maximum 1.69 ± 0.17 fold increase in PLD activity compared to the basal activity (n = 4-8) and VIP_2 displays a maximum 2.52 ± 0.32 fold increase in PLD stimulation (n = 7-13). There was no stimulation of [^{3}H]PtdBut production by VIP in untransfected CHO cells (unpublished observations). The coupling of the VIP receptors to adenylate cyclase (presumably via G_s), was also tested, as a control to ensure that the stable clones could couple effectively to their primary signal transduction pathway. The VIP_1 and VIP_2 receptors stimulated cAMP production with EC_{50}s for VIP of 25 ± 2 pM and 168 ± 7 pM respectively (Fig. 5.2). The basal concentration of cAMP in the stable CHO cells was typically 4.0 ± 0.2 pmol/assay, stimulation of the VIP_1 receptor evoked an increase in cAMP concentration to a maximum of 118 ± 5

pmol/assay compared to the 318 ± 7 pmol/assay found after VIP_2 receptor activation (both n = 6). In comparison to the VIP_1 receptor stable cell line there was 25 times more receptor present on the plasma membrane of the VIP_2-carrying CHO cells, in terms of pmol receptor per mg of protein, (Table 5.1).

Using competitive whole cell binding studies with displacement of [^{125}I]helodermin by non-radioactive helodermin, the B_{max} of VIP_1 and VIP_2 receptors was found to be 0.06 ± 0.01 and 1.5 ± 0.1 pmol/mg protein respectively. The level of the cAMP response from the VIP_1 and VIP_2 receptors would seem to reflect the difference in B_{max} of the two receptors, whereas the PLD activity downstream of either VIP receptor shows remarkable similarity. The contrasting nature of the signalling responses may be a function of the difference in coupling mechanisms in the cAMP and PLD transduction pathways and currently these differences remain unknown. However increases in cAMP concentration stimulated by direct stimulation of G_s with 1 or 5 μg/ml cholera toxin, or adenylate cyclase with 1 or 5 μM forskolin did not stimulate an increase in [^{3}H]PtdBut in CHO cells (unpublished observations). As demonstrated in previous chapters the coupling of GPCRs to PLD can involve the association with small G proteins, and ARF is a major activator of PLD in mammalian systems (Brown, H. A., et al. 1993, Cockcroft, S., et al. 1994, Hammond, S. M., et al. 1995), so a role for ARF in the activation of PLD by VIP receptors was investigated using brefeldin A (BFA), an inhibitor of guanine nucleotide exchange on ARF (Donaldson, J., et al. 1992). The results shown in Fig. 5.3 indicate for the first time that both VIP_1 and VIP_2 receptors do indeed couple to PLD via the small G protein ARF as shown by their sensitivity to BFA. The IC_{50} for BFA on PLD response elicited by

the VIP_1 and VIP_2 receptors was 44 ± 17 and 43 ± 18 μM respectively. Agonist-induced PLD activity via the VIP_2 receptor was significantly reduced at 50μM BFA or greater ($p<0.05$, n = 8-10), and by 100μM BFA or greater at the VIP_1 receptor (n = 4-9). There was 65 ± 15% inhibition of the VIP_1 receptor response at 200μM BFA, and similarly 61 ± 10% inhibition of the VIP_2 receptor-mediated response at the same concentration.

The activation of PLD by either VIP receptor as described above seems most unlikely to be downstream of increased inositol phosphate metabolism as there was no detectable increase in inositol phosphate production in the stable CHO cell lines, in response to agonist (C.J., MacKenzie , unpublished observations). There have been reports by other investigators of InsP responses of VIP_1 receptors in CHO cells (van Rampelbergh, J., et al. 1997). The discrepancy in observations is likely to represent differences due to clonal variation and method of measurement of inositol phosphates. The stimulation by 1 μM VIP of the VIP_1 receptor stably expressed in CHO cells, with a receptor density of ≈ 20 pmol/mg protein, lead to a modest 1.5 fold stimulation of InsP production, thus it appears that in CHO cell lines the stably expressed VIP receptor does not couple strongly to PLC (van Rampelbergh, J., et al. 1997).

PACAP receptor signalling in response to agonist

The investigation of PACAP receptor signal transduction with regard to activation of PLD and cAMP was carried out on stable transfectants of two splice variants of the PACAP receptor cloned from the rat (Hashimoto, H., et al. 1993, Hosoya, M., et al. 1993, Morrow, J. A., et al. 1993, Pisegna, J. R. and S. A. Wank 1993, Spengler, D., et al. 1993, Svoboda, M., et al. 1993). Activation of PLD by PACAP-38 investigated at $PACAP_{short}$ receptor and

$PACAP_{long}$ receptor splice variants; the long form receptor possessing the extra hop-1 cassette in its i3 loop (Fig. 5.4) Both PACAP receptor forms mediated activation of PLD in response to PACAP-38 in a potent, concentration-dependent manner. The $PACAP_{short}$ and $_{long}$ receptors stimulated a maximum increase in PLD activity of 1.92 ± 0.15 and 5.74 ± 0.46 fold increase over basal control. The difference in the capability of the two receptors to activate PLD was not reflected in the concentration of PACAP-38 needed to stimulate a half-maximal PLD response, both receptors exhibiting very similar EC_{50}s for PACAP-38 of 2.31 ± 1.19 nM for the short form and 4.16 ± 0.95 nM for the longer analogue (Fig. 5.5). The larger PLD response elicited by the $PACAP_{long}$ receptor was not a consequence of more $PACAP_{long}$ receptors on the cell surface compared to the short form receptor as there was more short variant expressed in the relevant CHO clone (9.2 ± 0.9 pmol/mg protein) than long variant (6.2 ± 0.1 pmol/mg protein) (Table 5.1). As with the VIP_1 and VIP_2 receptors the ability of the stable PACAP receptor clones to increase cAMP concentrations by coupling to adenylate cyclase was verified and as expected from their very similar B_{max} values, the PACAP receptors exhibited a similar capacity to increase cAMP levels. The EC_{50} values for cAMP production by the $PACAP_{short}$ and $_{long}$ variants were 27 ± 2 pM and 22 ± 2 pM respectively, with a maximum increase in cAMP concentration to 355 ± 9 pmol/assay for the $PACAP_{short}$ receptor (106 ± 3 fold over basal) and 330 ± 37 pmol/assay for $PACAP_{long}$ (81 ± 9 fold over basal), (Fig. 5.6). Evidence for the involvement of ARF in coupling the VIP_1 and VIP_2 receptors to PLD (as detailed above) suggests that small G proteins can play a role in the activation of PLD by the class II family of receptors. As shown for the first time in Figure 5.7 ARF also appears to be involved in the stimulation of PLD downstream of PACAP receptors. Pre-treatment of

CHO cells expressing the $PACAP_{long}$ form receptor with 25 μM BFA markedly reduced the activation of PLD in response to PACAP-38 ($p<0.05$), and BFA showed an IC_{50} of 41 ± 4 μM. In remarkable contrast the $PACAP_{short}$ receptor showed no significant decrease in its ability to activate PLD until 200 μM BFA was used to pre-treat the cells and this caused only a 36 ± 16% inhibition of enzymatic activity ($p<0.05$). The efficiency and potency of both PACAP receptors' coupling to adenylate cyclase is comparable and therefore it seems unlikely that the differences in PLD activation observed are downstream of such congruent cAMP signalling. The PACAP receptors under investigation in this study are known as efficient activators of IP metabolism (Spengler, D., et al. 1993, van Rampelbergh, J., et al. 1997) and in assays monitoring InsP breakdown by the receptors expressed in CHO cells, there was a concentration-dependent increase in inositol phosphates with a maximum increase over basal control of 5.6 ± 0.3 fold for the $PACAP_{long}$ form and 10.3 ± 0.4 for the $PACAP_{short}$ form and respective EC_{50} values for PACAP-38 of 8.1 ± 2.1 and 14.9 ± 2.6 nM (C.J., MacKenzie unpublished observations). The possibility that PLD activation by the PACAP receptors may be downstream of their efficient PLC activation in CHO cells was addressed using the selective PLC inhibitor U73122 (Figure 5.8). The activation of PLD by the $PACAP_{long}$ form receptor was relatively insensitive to U73122, with only 31 ± 5% inhibition of PACAP-38-induced PLD activity seen after 90 minute pre-treatment of cells with 20 μM U73122. The reverse was true of $PACAP_{short}$ which showed much greater sensitivity to U73122, with 64 ± 4% inhibition of PLD activity at 20 μM U73122 ($p<0.01$) and an IC_{50} for U73122 of 5 ± 1μM. These results suggest that although the two PACAP splice variants differ by only the 28 amino acid hop-1 cassette, the long form mainly employs an ARF-dependent mechanism to couple to PLD,

whilst the short form activates PLD largely via PLC, and perhaps as a consequence of a rise in intracellular Ca^{2+} or PKC activity. The PLD activity stimulated in COS 7 cells by the calcium ionophore, ionomycin, or the PKC activator phorbol 12,13-dibutyrate was insensitive to BFA (Mitchell, R., et al. 1998). Therefore the activation of PLD downstream of PLC activation would appear to represent a route for the stimulation of phosphatidylcholine breakdown distinct form that involving ARF. In studies using BFA on the $PACAP_{long}$ form and U73122 on the $PACAP_{short}$ form, the stimulated PLD activity was never completely inhibited and this may be a consequence of dual coupling to PLD. The receptors may employ two mechanisms for activation of PLD, for example the $PACAP_{long}$ form coupling to PLD via ARF (primary) and PLC (secondary), with $PACAP_{short}$ the order would be PLC (primary) and ARF (secondary).

Chimaeric VIP_2/PACAP receptors: activation of PLD in response to agonist

The important influence of i3 in the coupling of the $PACAP_{short}$ and $_{long}$ receptor subtypes to PLD, as shown by the distinctive routes of activation of PLD observed (despite equivalent levels of agonist binding) suggested that within the i3 of these receptors lie major determinants of G protein coupling. The i3 of GPCRs is accepted as one of the crucial domains controlling G protein coupling (reviewed recently by (Bourne, H. 1997, Gudermann, T., et al. 1996, Savarese, T. M. and C. M. Fraser 1992, Strader, C. D., et al. 1994, Wess, J. 1997)). The construction of chimaeric receptors carrying a mainly VIP_2 body but with its i3 replaced by that of the $PACAP_{short}$ or $_{long}$ receptor (as shown in Fig. 5.9) allowed us to study whether indeed the different i3 domains of the receptors were enough to switch the coupling behaviour of the receptor. The cDNAs for the

$VIP_2/PACAP_{short}$ (VP/4/7b/2.1c) and $VIP_2/PACAP_{long}$ (VP/2.1c) chimaeric i3 insert receptors were transiently expressed in COS 7 cells. The chimaeric receptors activated PLD in response to VIP to a similar level as observed with the VIP_2 receptor (Fig. 5.10), that is a 3.2 ± 0.2 fold increase over basal elicited by the $VIP_2/PACAP_{short}$ chimaeric receptor and a 2.5 ± 0.4 fold increase after $VIP_2/PACAP_{long}$ receptor stimulation. The EC_{50}s for VIP-induced PLD activation by the $VIP_2/PACAP_{short}$ and $_{long}$ chimaeric receptors were 2.5 ± 1.7 nM and 5.6 ± 3.9 nM respectively, values that are similar to the VIP_2 and $PACAP_{short}$ and $_{long}$ receptors expressed in CHO cells. The involvement of ARF in the coupling of the chimaeric constructs was investigated and as with the PACAP receptor splice variants expressed stably in CHO cells there was a dissimilar sensitivity to BFA. The $VIP_2/PACAP_{long}$ receptor activated PLD in a BFA-sensitive fashion and the $VIP_2/PACAP_{short}$ chimaeric construct mimicked the $PACAP_{short}$ receptor and was BFA-resistant in the stimulation of PLD (Fig. 5.11). The highest concentration of BFA used (200 μM) caused only 32 ± 16% inhibition of the $VIP_2/PACAP_{short}$ receptor stimulated PLD activity, compared to 84 ± 16% inhibition of the $VIP_2/PACAP_{long}$ receptor response where BFA displayed an IC_{50} of 43 ± 6 μM ($p<0.05$). Therefore the i3 of the $PACAP_{long}$ receptor as with the VIP_1 and VIP_2 receptors contains major determinants that dictate coupling of the receptor to PLD via ARF. In contrast the $PACAP_{short}$ i3 does not appear to possess the required motifs or does not have them in the correct conformation to elicit a strong association of the receptor with small G proteins in its activation of PLD. The activation of PLD after PACAP-38 binding to its receptor may be the result of receptor connection to various pathways, and the possession of either the hop-1, hop-2, hip or combination of these inserts allows the receptor to select a dominant

route. Alternative splicing of the genes coding for GPCRs introduces another level of variation and fine control of responses to agonist, as shown by the abolition of PLC stimulation in the rat and human PACAP receptors carrying the hip cassette or the analogous calcitonin receptors with a 16 amino acid insert in intracellular loop 1 (i1) (Houssami, S., et al. 1994, Moore, E. E., et al. 1995, Nussenzveig, D. R. and C. N. Thaw 1994, Pisegna, J. R. and S. A. Wank 1996, Spengler, D., et al. 1993)

5.4 DISCUSSION

Pathways to PLD activation

The results shown in Figures 5.1, 5.5 and 5.10 demonstrate for the first time that members of the class II family of receptors can potently activate PLD, and that this coupling may well be dependent on the small G protein ARF in the cases of the VIP_1, VIP_2 and $PACAP_{long}$ receptors. A requirement for ARF would point to an activation of PLD1 or PLD2 by these receptors in CHO and COS-7 cells (Hammond, S. M., et al. 1995, Hammond, S. M., et al. 1997, Lopez, I., et al. 1998). However ARF stimulated the human PLD2 enzyme only 2 fold, compared to the 20 fold stimulation of the hPLD1 enzyme (Lopez, I., et al. 1998) and furthermore the rodent PLD2 enzyme was not detectably stimulated by ARF (Colley, W. C., et al. 1997).

Receptor-stimulated cAMP production

The coupling of this family of receptors to the heterotrimeric G protein G_s with concomitant activation of adenylate cyclase and rise in intracellular cAMP levels is seen as one of the characteristics of this family of receptors

(Laburthe, M., et al. 1996, Segre, G. V. and S. R. Goldring 1993). The VIP_1, VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors stably expressed in CHO cells all elicited increases in intracellular cAMP concentration in a potent and efficacious manner as shown in Figures 5.2 and 5.6. In all four receptors studied, the EC_{50} values for cAMP production were lower than the IC_{50} values for displacement of bound radiolabel, this amplification of the cAMP response after the activation of a small population of the available receptor sites suggests the presence of spare receptors for this response (Kenakin, T. P. 1984). An identical situation was reported by Ciccarelli *et al.* who also expressed the $PACAP_{short}$ and $PACAP_{long}$ (hop-1) receptors in CHO cells, and described lower EC_{50} values compared to K_d values for binding of tracer (Ciccarelli, E., et al. 1995). In another study, Ciccarelli *et al.* described the magnitude of the cAMP response as B_{max}-dependent, therefore taking into account the existence of receptor reserve, increasing the concentration of VIP_1 receptors expressed in CHO cells increased the maximal cAMP response that was achieved (Ciccarelli, E., et al. 1994). The results obtained from the VIP_1, VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors in this study could well be in agreement with the observation reported by Cicarelli *et al.*: the VIP_2 receptor stimulated a maximal cAMP concentration 2.7 fold larger than that induced by the VIP_1 receptor, matching the significantly greater concentration of receptor present. The PACAP receptors with their similar B_{max} values showed equivalent capacity to increase cAMP in response to PACAP-38.

Receptor coupling to phosphoinositide hydrolysis

The stimulation of phosphoinositide metabolism is observed for many receptors in class II such as the cloned calcitonin, glucagon, PTH/PTHrP and glucagon-like peptide-1 receptors (Abousamra, A. B., et al. 1992,

Chabre, O., et al. 1992, Force, T., et al. 1992, Wheeler, M. B., et al. 1993) and secretin increases inositol phosphate hydrolysis in native pancreatic tissue (Trimble, E. R., et al. 1987). VIP has also been reported to increase the breakdown of inositol phosphates in rat astroglia (Fatatis, A., et al. 1994), and the mouse VIP_2 receptor stimulated calcium-activated chloride currents, which implicates a coupling to PLC (Inagaki, N., et al. 1994). There is little knowledge of the range of transduction abilities of the class II receptors. PACAP receptors can stimulate inositol phosphate breakdown and cytoplasmic calcium concentrations not only in transfected LLC-PK1 (Spengler, D., et al. 1993) and CHO cells (Delporte, C., et al. 1995, van Rampelbergh, J., et al. 1997) but also in normal pituitary gonadotropes (Rawlings, S. R., et al. 1993, Rawlings, S. R., et al. 1994), in rat gonadotrope-derived αT3-1 cells (Schomerus, E., et al. 1994) in PC12 cells (Deutsch, P. J. and Y. Sun 1992) and in the neuroblastoma cell line NB-OK-1 (Delporte, C., et al. 1993), presumably via coupling to G_q. In our CHO cells stably expressing the $PACAP_{short}$ and $_{long}$ receptor, there was a strong activation of inositol phosphate production in response to PACAP-38, with a 10.3 ± 0.4 and 5.6 ± 0.3 fold increase in inositol phosphate production over basal activity respectively. The slightly higher expression level of $PACAP_{short}$ receptors, compared to the $_{long}$ form as seen in Table 5.1 probably determines the greater InsP response as there is little evidence for spare receptors in the activation of PLC.

Both VIP_1 receptors stably expressed in CHO cells (Sreedharan, S. P., et al. 1994, van Rampelbergh, J., et al. 1997) and VIP_2 receptors transiently expressed in COS-7 cells (MacKenzie, C. J., et al. 1996) have been shown to couple to PLC in a pertussis toxin (PTx)-sensitive manner, suggesting the involvement of a member of the $G_{i/o}$ family of G proteins. The

investigators report that in these systems PACAP receptors couple to PLC in a PTx-insensitive manner. Contrary to the results published by Van Rampelbergh *et al.* we could detect no rise in inositol phosphate breakdown above basal activity by our agonist-treated VIP_1 receptor carrying clones, or by VIP_2 receptors also expressed in this cell line (C.J., MacKenzie unpublished observations). The difference may be a result of clonal difference, Robberecht's group observed a maximal 1.5 fold stimulation of InsP breakdown by the VIP_1 receptor, compared to control, when expressing 20 pmol/mg receptor protein (van Rampelbergh, J., et al. 1997). In comparison there was 0.06 ± 0.01 and 1.5 ± 0.1 pmol/mg VIP_1 and VIP_2 receptor respectively expressed at the plasma membrane of the CHO clones used in this study (Table 5.1). Differences in the techniques used to measure inositol phosphate accumulation may also contribute to this disparity. Interestingly, at the same B_{max} values of 20 pmol/mg protein, VanRampelbergh *et al.* reported a 3 fold stimulation of InsP production by the $PACAP_{short}$ receptor, expressed in CHO cells, demonstrating a more efficacious coupling of PACAP receptors to phosphoinositide breakdown. However this response is still much lower than the phosphoinositide breakdown observed after stimulation of PACAP receptors in our system.

Further receptor-evoked signal transduction pathways

There have been scattered reports of activation of other signal transduction pathways by VIP and PACAP, although some of these may be downstream of the established responses described above: VIP has been described to induce activation of nitric oxide synthase (NOS) in gastric smooth muscle cells via G_{i1-2} (Murthy, K. S. and G. M. Makhlouf 1994). VIP induces activation of the non-receptor tyrosine kinase $p59^{fyn}$ in murine thymocytes (Xin, Z. C., et al. 1997), subnanomolar concentrations

of VIP stimulate the translocation of PKC alpha, delta and zeta isoforms to the nucleus of neonatal rat astrocytes (Olah, Z., et al. 1994). PACAP has been shown to activate extracellular signal-related/mitogen-activated protein kinase (ERK/MAPK) in cerebellar granule neurons (Villalba, M. and L. Journot 1997) and PC12 cells (an important pathway in these cells required for differentiation into a sympathetic-like neuron (Barrie, A. P., et al. 1997)). Similarly Zhong reported a PACAP-like neuropeptide to activate the Ras/Raf pathway in Drosophila (Zhong, Y. 1995). Finally the involvement of a wortmannin-sensitive signal transduction pathway in VIP/PACAP-induced insulin release from a pancreatic-derived HIT-T15 β-cell line was reported by Straub and Sharp. VIP and PACAP stimulated this activity with a pharmacological profile characteristic of the VIP_2 receptor (Straub, S. G. and G. W. G. Sharp 1996), which is strongly expressed in normal pancreatic tissue (Usdin, T. B., et al. 1994). Therefore one of the characteristics of this family of receptors would appear to be the ability to couple to multiple second messenger pathways. However in contrast to the rhodopsin family of receptors where there is a detailed understanding of many of the receptor-mediated transduction mechanisms, and their physiological relevance, the signalling capabilities of the class II receptor family are still largely to be explored.

Receptor determinants and motifs

The activation of PLD by the VIP and PACAP receptors is similar to the activation of PLC, in that although the efficacy of the response is weak compared to the increase in cAMP concentration, the concentrations of agonist required to achieve half-maximal stimulation are low (in the nM range) and within physiological limits. The activation of PLD by both VIP receptors and the $PACAP_{long}$ form has been implicated as being ARF-

dependent (Figures 5.3 and 5.7). Moreover the $PACAP_{short}$ receptor appears to stimulate PLD via a coupling to PLC and is not sensitive to the ARF inhibitor BFA. An individual GPCR, when activated by its ligand, can recognise and activate only a limited set of the structurally related G-proteins. Extensive investigations with receptor peptides, chimaeras and site-directed mutants have shown that the i3 sequence determines $G\alpha\beta\gamma$ selectivity more often than does i2, or the C-terminal tail and that i1 rarely determines specificity and that loop sequences near TMDs are especially important determinants of specificity (reviewed in (Gudermann, T., et al. 1996, Strader, C. D., et al. 1995, Strader, C. D., et al. 1994, Wess, J. 1997)). On the other hand the calcitonin receptor i1 is reported to be involved in control of receptor coupling to phosphoinositide breakdown (Houssami, S., et al. 1994, Nussenzveig, D. R. and C. N. Thaw 1994), perhaps reflecting a slightly different mechanism of class II receptor coupling to G proteins.

The direct interaction of class II receptors with small G proteins is yet to be proven, although as we have reported (Mitchell, R., et al. 1998) and in previous chapters, the rhodopsin family of receptors can associate with small G proteins. It would thus be logical to assume that the receptor can discriminate between different monomeric G proteins in a manner analogous to its interaction with the α subunits of heterotrimeric G proteins. Following this line of reasoning, the BFA-sensitive receptors therefore may possess structural determinants (absent in the $PACAP_{short}$ receptor) that allow them to couple to PLD via ARF, and as described above, the i3 loop of GPCRs is likely to be most important domain in terms of G protein selectivity. The differences in the coupling of the $PACAP_{short}$ and $_{long}$ form receptors to PLD support this idea; their divergent ability to activate PLD can only be explained by their different i3

loop structures. Therefore the extra 28 amino acid hop-1 cassette, present in the $PACAP_{long}$ receptor provides the interfaces required to allow the receptor to couple to ARF, in a similar way to that available for the VIP_1 and VIP_2 receptors, but not for the $PACAP_{short}$ receptor.

The analysis of the signalling capabilities of chimaeric receptors comprised of the VIP_2 receptor with part of TM5, the whole of the i3 loop and part of TM6 replaced with that of either the $PACAP_{short}$ and $_{long}$ receptors showed explicitly that the i3 of the PACAP receptor does indeed determine the coupling of the receptor to PLD via ARF. Only this domain was transferred into the chimaeric receptor and it was sufficient to obtain a BFA-sensitive ability to activate PLD. The VIP_2/PACAP chimaeric receptors activate PLD in an VIP-dependent manner, to a similar maximal extent as seen for the wild type VIP_2 receptor expressed in CHO cells, thereby showing that the i3 swap does not interfere with ligand binding and activation of the receptor, but that the i3 as shown in Figure 5.11 quite selectively determines the BFA-sensitivity of the PACAP receptors. A somewhat analogous situation has been reported with the presence of two isoforms of the dopamine D_2 receptor. D_{2short} and D_{2long} receptors are generated by alternative splicing of the same gene, and a 29 amino acid insert is included in the i3 of the D_{2long} receptor. The extra 29 amino acids allow the longer dopamine receptor to couple to the inhibitory G protein $G\alpha_{i2}$, which is not seen for the D_{2short} receptor (Guiramand, J., et al. 1995). Also a 16 amino acid insert in the i1 of the calcitonin receptor abolishes the receptor's ability to stimulate phosphoinositide breakdown without affecting the adenylate cyclase activation by the receptor (Houssami, S., et al. 1994, Nussenzveig, D. R. and C. N. Thaw 1994). Thus the alternative splicing of receptor gene transcripts can allow another level of signalling

complexity to be introduced, and a more subtle control of cellular activity to be achieved.

Importance of basic amino acid residues in signal transduction motifs

Work done by Nishimoto's group has implicated a crucial role for basic amino acid residues in the i3 of GPCRs for the functional coupling of receptors to G-proteins. Using peptide sequences derived from the α_2-adrenergic receptor, Nishimoto reported that peptides containing numerous basic amino acids spaced throughout the peptide and ending with a BBXXB or BBXB (where X = any amino acid, and the last B can be a basic or aromatic amino acid) were strong activators of $G_{i/o}$ (Ikezu, T., et al. 1992). The importance of a cluster of basic amino acids situated in i3 (approximately 14-18 amino acids from TMD 6) in the receptor coupling to G proteins were suggested by work done on the m1 muscarinic receptor coupling to PLC. This region was suggested to be important in G protein activation, but not important for regulation and selectivity (Burstein, E. S., et al. 1995). Similarly substitution and deletion of basic amino acids in the i3 of the M_1 and M_3 muscarinic receptors or peptides derived from the i3 of the α_2-adrenergic receptor, close to the i3/TMD 6 junction, reduce receptor coupling to $G_{q/11}$ and G_o respectively (Blin, N., et al. 1995, Lee, C. H., et al. 1993, Wade, S. M., et al. 1996). The VIP_2 receptor contains a classical BBXXB motif at the i3/TM6 junction, whereas both the VIP_1 and $PACAP_{long}$ form receptors contain the spaced base motif seen in the α_2-adrenergic receptor and the cluster of basic amino acids present 14-18 amino acids upstream of TM6, as detailed by the work of Burstein *et al.*. (Burstein, E. S., et al. 1995). The presence of the hop-1 cassette provides the clustered motif of basic amino acids implicated in G protein coupling by the work detailed above. The $PACAP_{short}$ receptor has no classical or

spaced base motif present in its i3 and this may be a reason for its lack of coupling to PLD via ARF. The role of basic amino acids in receptor-G protein coupling is based on studies of the class I family of receptors, and as detailed work on the domains involved in G protein coupling in the class II family has yet to be undertaken, there is no consensus of opinion regarding receptor regions crucial to G protein coupling.

Control of receptor coupling to adenylate cyclase/PLC activation

The fold increase in cAMP concentration and phosphoinositide breakdown observed downstream of VIP and PACAP receptor activation in this study is dependent on the concentration of receptor present, as reported by other groups (Ciccarelli, E., et al. 1994, van Rampelbergh, J., et al. 1997) and the activation of PLD by both VIP receptors was similarly dependent on B_{max}. However the $PACAP_{long}$ receptor elicited a 3 fold stronger PLD response compared to the $PACAP_{short}$ receptor, despite the presence of 1.5 times more $_{short}$ splice variant receptor expressed on the surface of its stable CHO clone. The $PACAP_{short}$ receptor appears to activate PLD via activation of PLC as shown by the U73122-sensitive nature of the response (Figure 5.8). The activation of PLC would lead to the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, M. J. 1993); IP_3 induces elevations in the cytoplasmic calcium concentration, and Ca^{2+}/DAG activate PKC (Nishizuka, Y. 1992) which has been shown to be a positive regulator of PLD (Hammond, S. M., et al. 1995, Hammond, S. M., et al. 1997). The activation of PLD by PKC alone would reflect an indirect and perhaps weaker pathway for coupling of the receptor to phosphatidylcholine hydrolysis, thus explaining the poorer stimulation of PLD activity by the $PACAP_{short}$ receptor. The ability of the VIP and PACAP receptors to stimulate increases in cAMP

concentration and phosphoinositide breakdown in CHO cells are very similar and the presence of the hop-1 cassette does not appear to significantly change the cAMP responses elicited by the $PACAP_{long}$ form as compared to the short form. By analogy with the rhodopsin family of receptors, the determinants for receptor coupling to the G_s/adenylate cyclase and G_q/PLC systems are most probably found within the cytoplasmic loops present and experiments on other members of the class II family of receptors indicate a role for i1 and i2 in receptor coupling to cAMP and phosphoinositide metabolism, in addition to the i3.

Heller *et al.* reported that substitution of the basic arginine residue with glycine in the glucagon-like peptide-1 receptor, at the C-terminal end of i3 (Ci3), almost abolished the cAMP response (Heller, R. S., et al. 1996). Chicchi *et al.* reported the importance of the KLR motif in the N terminal part if the glucagon receptor i3 loop (Ni3) which, when deleted, lead to a 39% decrease in the cAMP response (Chicchi, G. G., et al. 1997). A homologous KLQ motif exists in the PACAP receptors and KLR and KLT motif in VIP_1 and VIP_2 receptors respectively. Mutation of the equivalent amino acids in these receptors has not been reported, but the effect on cAMP responses might be expected to mimic the results seen in the glucagon receptor.

Constitutive activation of the human VIP_1 receptor was observed after the mutation of a highly conserved histidine at position 178 to arginine at the TM2/i1 junction. The same constitutively-activating mutation in the PTH/PTHrP receptor is associated with Jansen-type metaphyseal chondrodysplasia (Gaudin, P., et al. 1998). These reports are consistent with the idea that basic amino acids can have profound effects on receptor function. In mutagenesis experiments on the PTH/PTHrP receptor Iida-

Klein*et al.* found that mutation of lysine 319 in i2 to glutamate significantly reduced receptor coupling to phosphoinositide breakdown to 27 ± 13% of wild type, without affecting the activation of adenylate cyclase. The conservative replacement of the positively charged lysine 319 with a positively charged arginine had no effect on receptor signal transduction abilities (Iida-Klein, A., et al. 1997), emphasising the importance of basic amino acids in all aspects of receptor-G protein coupling.

Recently the phosphorylation of the secretin receptor by G protein-coupled receptor kinases (GRKs), which lead to desensitisation of the receptor-mediated cAMP response was reported (Shetzline, M. A., et al. 1998), The results of the study on the secretin receptor expressed in HEK 293 cells suggest that receptors of the class II family of GPCRs can be regulated by mechanisms similar to those of the rhodopsin (class I) family.

Physiological significance of VIP/PACAP-evoked PLD activity

The physiological significance of activation of PLD by the VIP and PACAP receptors, as with any receptor activation of the enzyme, is unclear. However there is significant overlap in the consequences of VIP/PACAP stimulation and PLD activation. Both VIP and PACAP can act as potent secretagogues; indeed PACAP activity in the pituitary is proposed to be that of a hypophysiotrophic factor (Rawlings, S. R. and M. Hezareh 1996). To expand on that argument, PACAP immunoreactivity is detected in hypothalamic neurons projecting to the external zone of the median eminence of the hypothalamus, it is released into the hypophysial portal blood system, can bind to specific receptors detected within the pituitary and it modulates anterior pituitary cell activity, including the release of hormones such as luteinizing hormone (LH), follicle-stimulating

hormone (FSH), growth hormone (GH) and prolactin from normal anterior pituitary cells (reviewed in (Rawlings, S. R. and M. Hezareh 1996)). The $PACAP_{short}$ and $PACAP_{long}$ receptors (carrying the hop cassette), are the dominant forms of the PACAP receptors in the anterior pituitary (Rawlings, S. R., et al. 1995, Spengler, D., et al. 1993), although all forms of the PACAP receptor are observed. PACAP stimulates the release of LH, GH and prolactin from rats *in vivo* (Jarry, H., et al. 1992, Leonhardt, S., et al. 1992, Nagy, G. M., et al. 1993, Yamamuchi, K., et al. 1995). Moreover PACAP has been observed to stimulate luteinizing hormone release from normal gonadotrophs *in vitro,* in a manner that was not blocked by a VIP antagonist, therefore suggesting the release was mediated by binding to the PACAP receptor (Culler, M. D. and C. S. Paschall 1991). VIP stimulates the release of prolactin from the pituitary (Reichlin, S. 1988), and also PACAP and VIP can stimulate the release of (GH) from the somatotroph-like GH_3 cell, by acting on VIP_2 receptors (Murakami, Y., et al. 1995, Propato-Mussafiri, R., et al. 1992). VIP binding to the VIP_2 receptor in mouse pancreatic β cells in the presence of glucose augments the release of insulin and PACAP is proposed to function as a non-cholinergic neurotransmitter, stimulating catecholamine secretion from the adrenal medulla (Przywara, D. A., et al. 1996).

Thus VIP and PACAP both regulate the secretion of hormones and PLD has been proposed to play an important role in the regulation of secretory vesicles and membrane trafficking in mammalian cells (Bowman, E. P., et al. 1993, Brown, H. A., et al. 1993, Cockcroft, S., et al. 1994, Kahn, R. A., et al. 1993, Liscovitch, M. and L. C. Cantley 1995). Recently PLD1 was reportedly localised to secretory granules in COS-1, RBL-2H3 and neutrophils and upon agonist stimulation translocated to the plasma

membrane (Brown, F. D., et al. 1998, Morgan, C. P., et al. 1997). Furthermore ARF6 was also found to translocate from secretory granules to the plasma membrane in chromaffin cells with a concomitant activation of PLD (Caumont, A.-S., et al. 1998, Galas, M.-C., et al. 1997). The inhibition of this translocation with a synthetic ARF6 peptide blocked both PLD activation and catecholamine secretion in stimulated cells (Caumont, A.-S., et al. 1998, Galas, M.-C., et al. 1997). Thus the secretion of hormones may depend on the stimulation of PLD and its movement from the secretory granules within the cell to the plasma membrane. Thus ARF-dependent PLD activation may well be implicated in the control of secretion of prolactin, growth hormone and insulin and other hormones in response to VIP and PACAP. The regulation of secretory responses by VIP and PACAP receptors, through PLD, may involve the control of both the coatomer and clathrin-mediated secretory systems, at the trans-Golgi network, endosomal compartments and the plasma membrane.

VIP and PACAP both promote neuronal survival and mitosis (DiCicco-Bloom, E. and P. J. Deutsch 1992, Pincus, D. W., et al. 1990) and PLD hydrolysis of PC results in the production of two mitogenic lipids: PA and its metabolite lysoPA produced by the actions of a phospholipase A_2. PA has been implicated in the stimulation of PKC-ζ (Limatola, C., et al. 1994, Nakanishi, H. and J. H. Exton 1992), PLC-γ (Jones, G. A. and G. Carpenter 1993) and PIP-5K as described above (Moritz, A., et al. 1992), but perhaps more importantly, PA also regulates the activity of Ras, which plays a key role as a molecular switch in the signal transduction pathways that are associated with cell proliferation, differentiation and neoplasia (Barbacid, M. 1987). The function of Ras in mammalian cells is regulated by its

interaction with a cytoplasmic GTPase activating protein (GAP) that stimulates its conversion to the inactive form (Trahey, M. and F. McCormick 1987). The active form of Ras activates other proteins including Raf, which in turn activates $p42^{MAPK}$ and $p44^{MAPK}$, culminating in the activation of transcription factors, implicated in control of cell growth (Pelech, S. L. and J. S. Sanghera 1992). PA binds to and inhibits the $p21^{ras}$ GTPase activating protein (GAP) (Tsai, M., et al. 1991), Moreover PA also binds to a GTPase inhibitory protein, increasing its activity (Tsai, M., et al. 1990). Thus the action of PA is to promote the GTP-bound active form of Ras, and enhanced Ras activity leading to an increase in MAP kinase activity and this scheme may explain the potent effect PA has on cellular proliferation (Tsai, M., et al. 1989, Tsai, M., et al. 1989). Also it has been observed that PA binds to a site on Raf and enables it to translocate to the membrane where it becomes active (Ghosh, S., et al. 1996). The effects of lysophosphatidic acid are mediated by its binding to a specific cell surface GPCR (Moolenaar, W. H., et al. 1997, van der Bend, R. L., et al. 1992). There are reports of the isolation of the cDNA for an lysoPA-specific receptor from mouse, human and *Xenopus* oocytes (An, S., et al. 1998, Dickens, A. S., et al. 1997, Guo, Z., et al. 1996, Hecht, J. H., et al. 1996), coupled to G proteins of the G_i and G_q families. Lysophosphatidic acid stimulates mitogenesis in numerous cells types and this mitogenic effect is inhibited by pertussis toxin, suggesting the involvement of G_i, and there is much evidence that lysoPA stimulates the Ras/MAP kinase pathway (Hordijk, P. L., et al. 1994, Howe, L. R. and C. J. Marshall 1993, Kumagai, N., et al. 1993, McLees, A., et al. 1995, Moolenaar, W. H. 1995). The cellular actions of PA action may be the outcome of its breakdown, by a PA-specific PLA_2, first described in platelets by Billah *et al.* (Billah, M. M., et al. 1981, Lapetina, E. G., et al.

1981), and a form of this enzyme purified form rat brain described by Thomson *et al.*. (Thomson, F. J. and M. A. Clark 1995). Recently a PA-specific PLA_1 enzyme was purified and cloned, with abundant expression in bovine brain and testis (Higgs, H. N. and J. A. Glomset 1996, Higgs, H. N., et al. 1998). Therefore part of the actions of PA could perhaps be ascribed to the production of lysoPA and its consequences. As discussed previously, PACAP has been shown to activate the MAP kinase pathway in neuronal and PC12 cells (Barrie, A. P., et al. 1997, Villalba, M. and L. Journot 1997). Therefore lysoPA and PA both have potent effects on cell growth and the ability of VIP and PACAP to stimulate mitosis and survival in neuronal cells may be a consequence of PLD activation and generation of the mitogenic lipid mediators as described.

In summary this study is the first explicit demonstration that members of the class II (secretin/calcitonin) family of GPCRs can prominently activate PLD, downstream of receptor coupling to PLC or the small G protein ARF. The lack of significant sequence homology (<12%) between the class I and II families of receptors but apparent ability to activate many of the same signal transduction pathways (and as recently observed, regulation by the same receptor kinases (Shetzline, M. A., et al. 1998)), points to the importance of the general seven transmembrane receptor conformation (Baldwin, J. M. 1993, Baldwin, J. M. 1994, Donnelly, D. 1997). The conformation and recognition sites found within the VIP_1, VIP_2 and $PACAP_{long}$ receptors allow them to couple tightly to ARF and thus activate PLD in a BFA-sensitive manner. However the $PACAP_{short}$ receptor with its similar but distinct structure couples to PLD primarily through PLC and probably the activation of a PKC isoform. The small differences in PACAP receptor structure leading to divergent mechanisms

for activation of PLD mirror the differences observed in the mechanism of PLD activation by the wild type and mutant GnRH receptors, discussed in Chapter 4. The receptor association with G proteins requires further exploration, the use of chimaeric receptor constructs described here could be extended to isolate the specific G protein recognition sites within the PACAP receptor. Analogous studies of the rhodopsin family of receptors have made extensive use of chimaeric receptor constructs, and deletion and/or mutagenesis of amino acid residues within domains crucial to G protein coupling, such as the cytoplasmic loops and N-terminal portion of the receptor carboxy terminal tail (Gudermann, T., et al. 1996, Strader, C. D., et al. 1995, Strader, C. D., et al. 1994, Wess, J. 1997). The role of other PLD activators such as Rho family proteins, PKC and PIP_2 in VIP/PACAP-stimulated PC hydrolysis are potentially similar to that observed with class I receptor-induced activation, and provide obvious targets for further investigation.

Figure 5.1

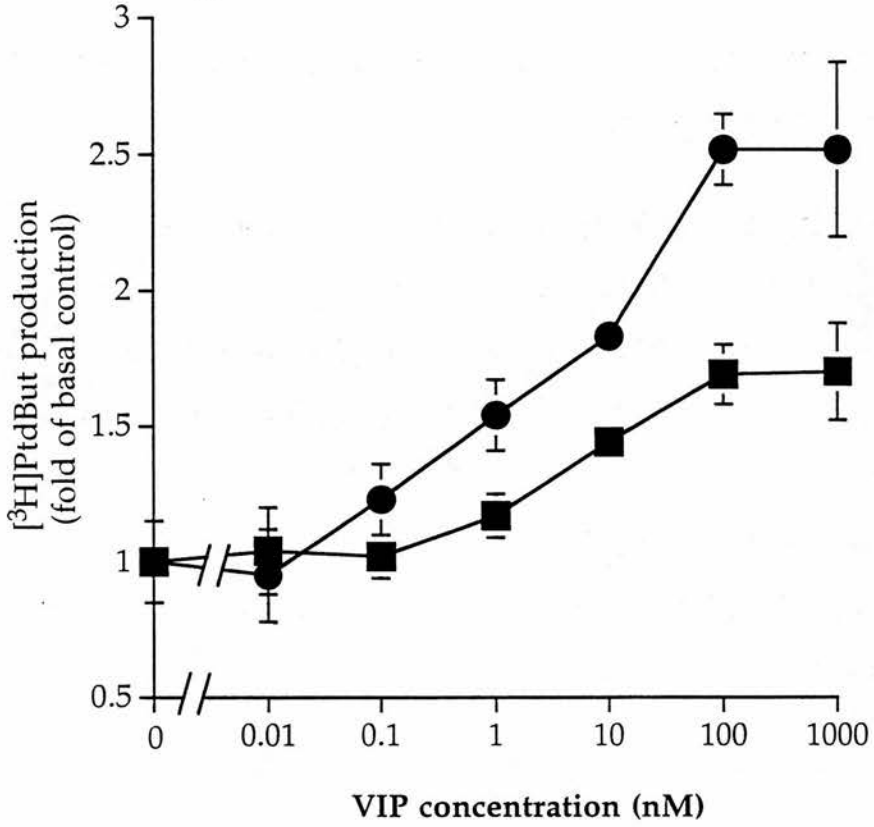

3
2.5
2
1.5
1
0.5
[3H]PtdBut production
(fold of basal control)
0
0.01
0.1
1
10
100
1000
VIP concentration (nM)

Figure 5.2

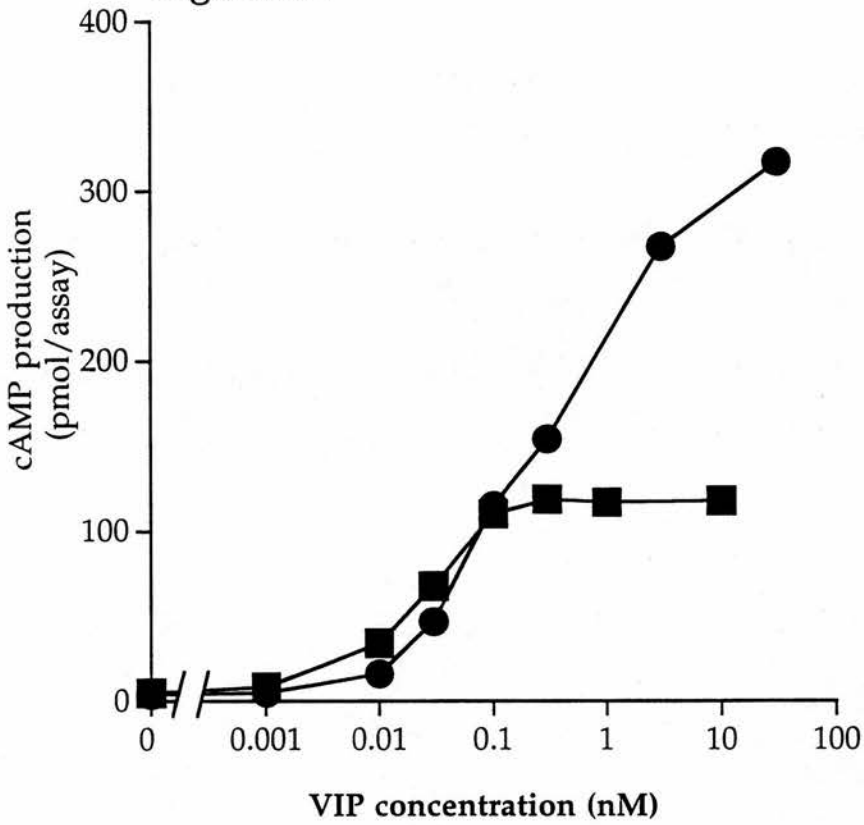

400
300
200
100
0
cAMP production
(pmol/assay)
0
0.001
0.01
0.1
1
10
100
VIP concentration (nM)

Table 5.1 **Concentration of cell surface receptors stably expressed in CHO cells and IC_{50} for relevant displacing peptide**

Receptor	B_{max} (pmol/mg protein)	IC_{50} (nM)
VIP_1	0.06 ± 0.01	helodermin 0.89 ± 0.09
VIP_2	1.5 ± 0.1	helodermin 4 ± 0.36
$PACAP_{short}$ form	9.2 ± 0.9	PACAP-27 13.2 ± 1.8
$PACAP_{long}$ form	6.0 ± 0.1	PACAP-27 6.2 ± 2.1

Whole cell binding of [^{125}I]helodermin to the VIP_1 and VIP_2 receptors and [^{125}I]PACAP-27 to the $PACAP_{short}$ and $PACAP_{long}$ receptors expressed stably in CHO cells. The IC_{50} value was determined by non-linear curve fitting (P. Fit) as described in Materials and Methods. Values are means ± SEM. (n = 6)

Figure 5.3

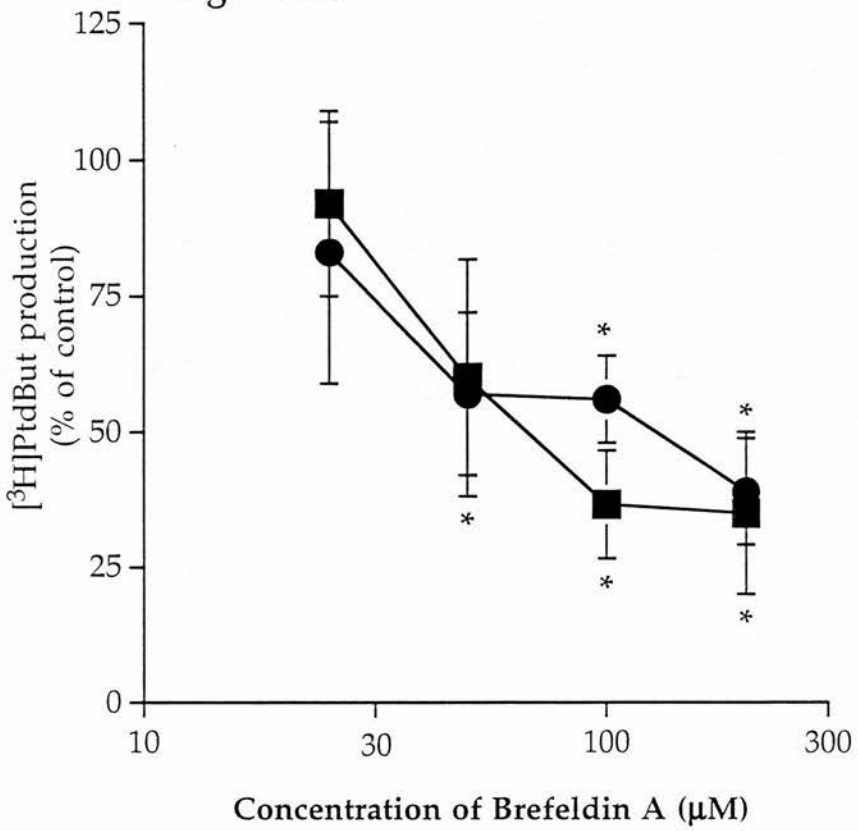
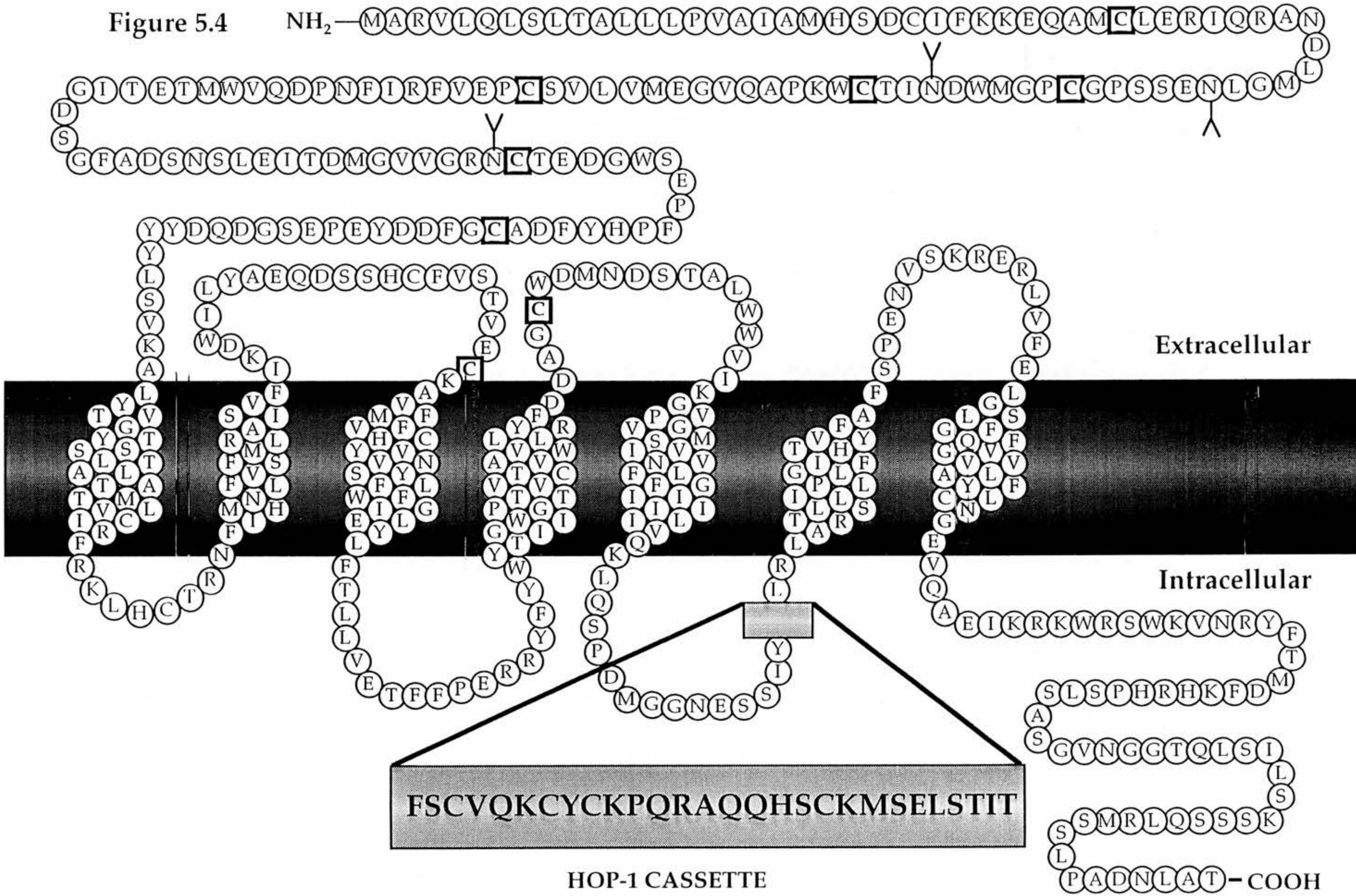
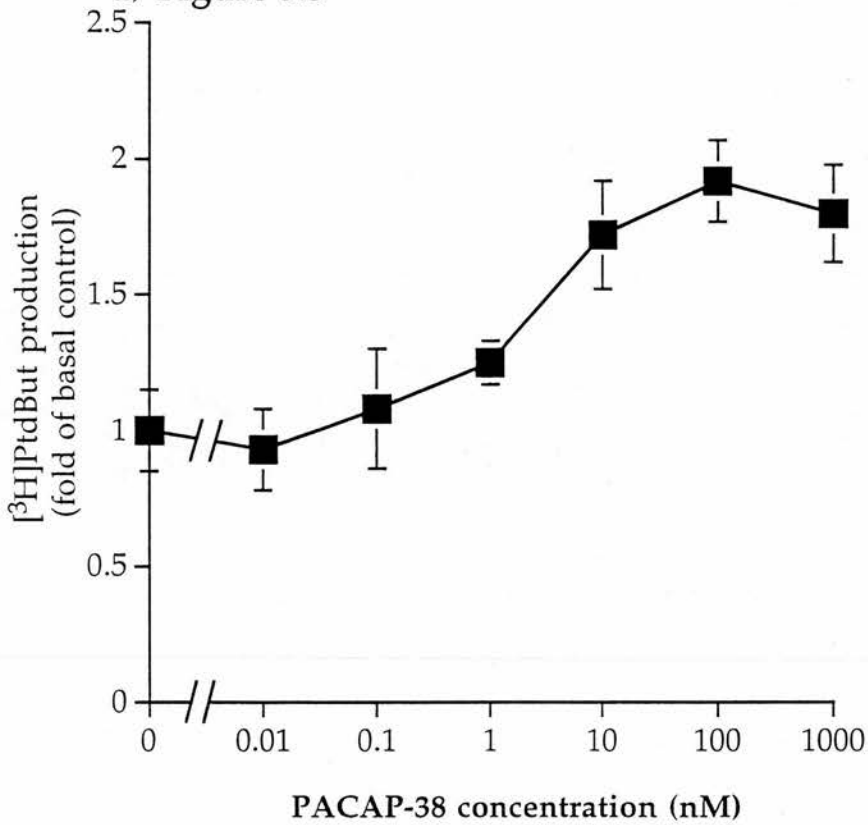

125
100
75
50
25
0
[3H]PtdBut production
(% of control)
10
30
100
300
Concentration of Brefeldin A (μM)
*
*
*
*

Figure 5.4


NH2
Extracellular
Intracellular
FSCVQKCYCKPQRAQQHSCKMSELSTIT
HOP-1 CASSETTE
COOH

a) Figure 5.5



b) Figure 5.5

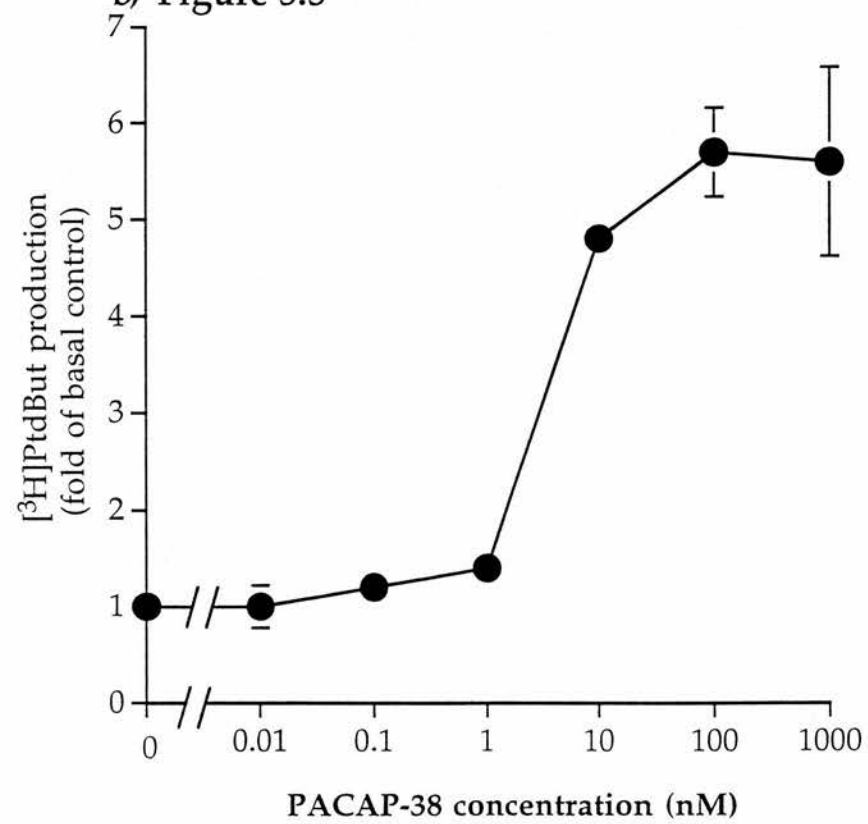


Figure 5.6

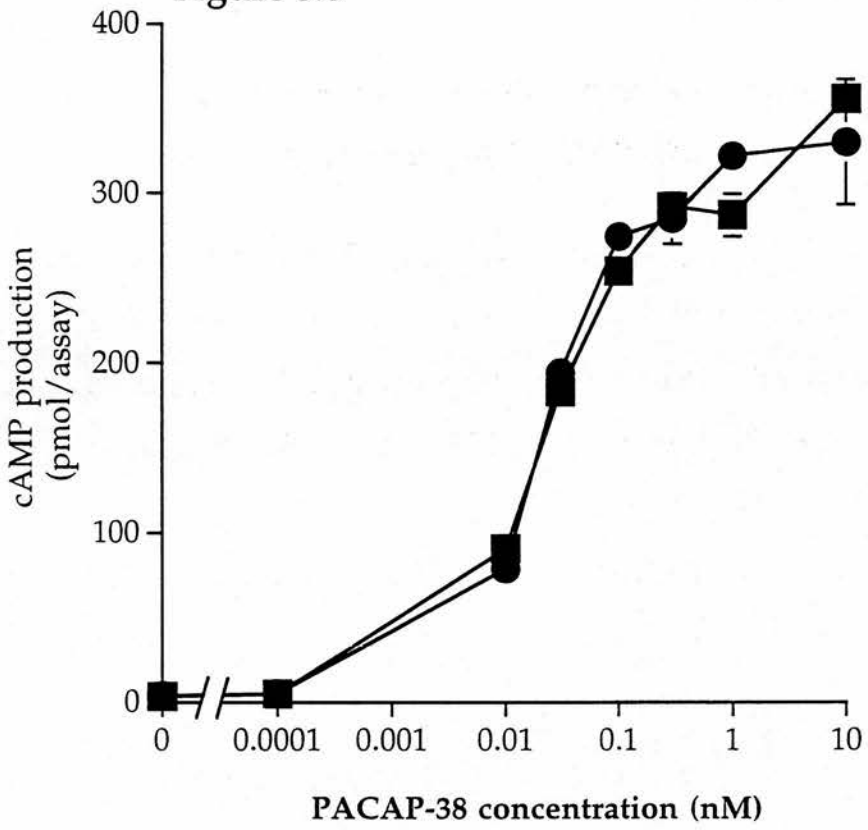

400
300
200
100
0
cAMP production
(pmol/assay)
0
0.0001
0.001
0.01
0.1
1
10
PACAP-38 concentration (nM)

Figure 5.7

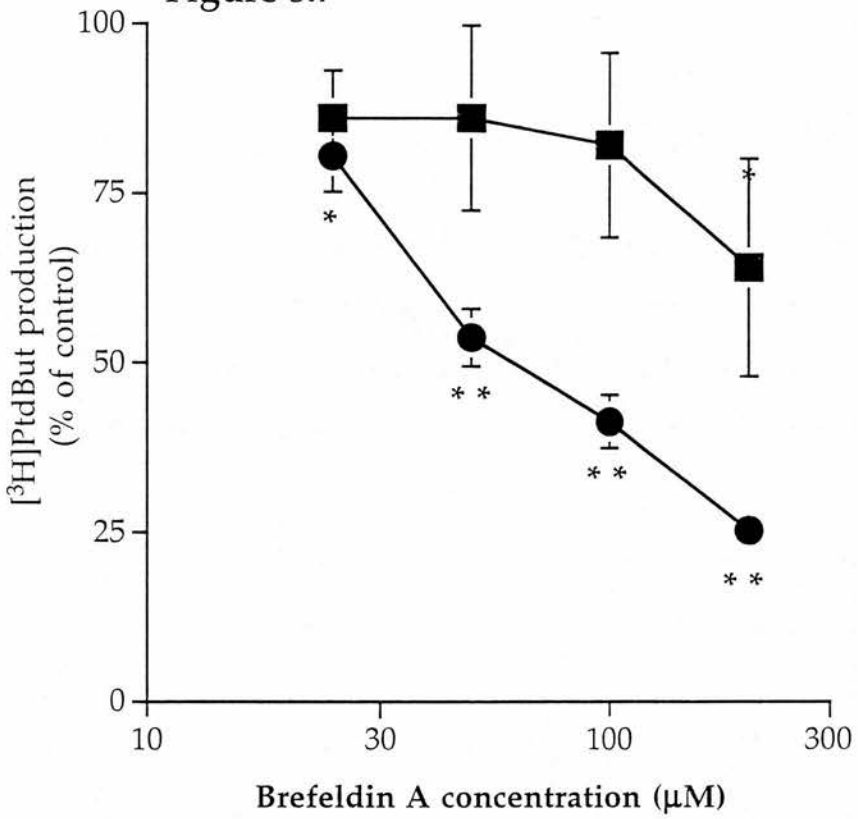

100
75
50
25
0
[3H]PtdBut production
(% of control)
10
30
100
300
Brefeldin A concentration (μM)
*
* *
* *
* *
*

Figure 5.8

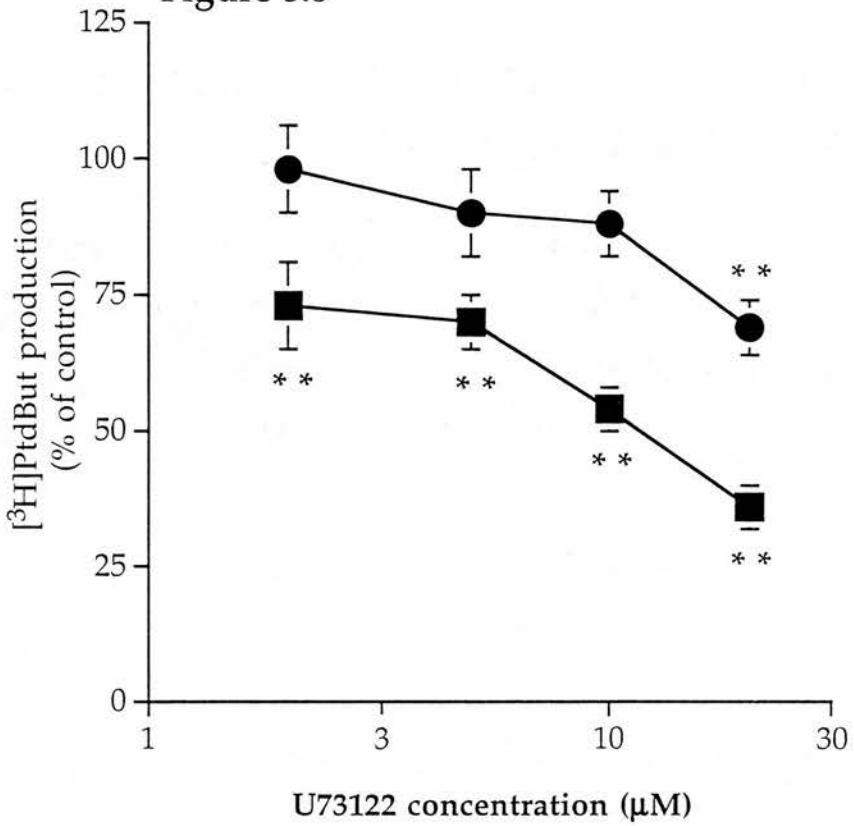

125
100
75
50
25
0
[3H]PtdBut production
(% of control)
1
3
10
30
U73122 concentration (μM)
* *
* *
* *
* *
* *

Figure 5.9

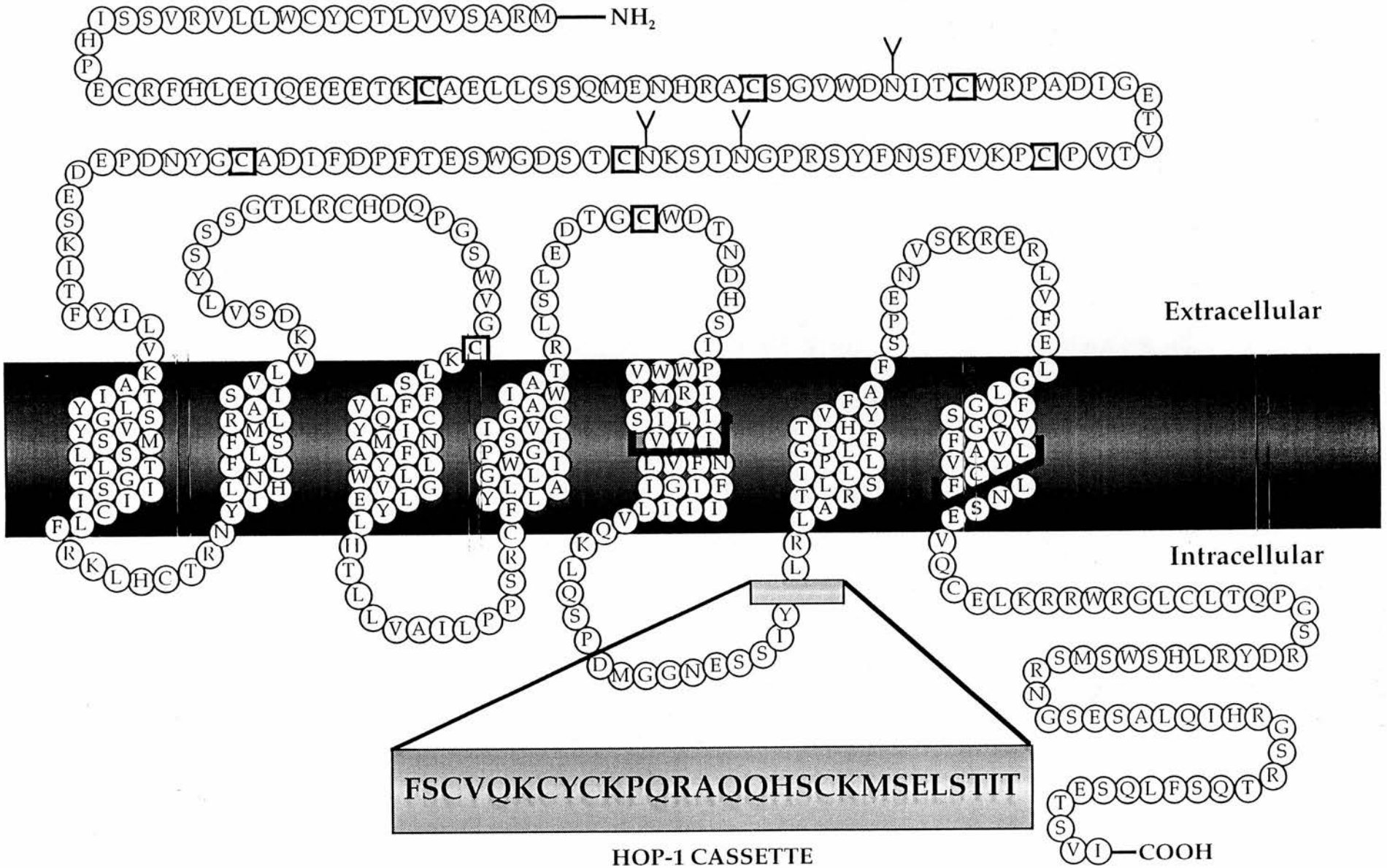

NH2
Extracellular
Intracellular
FSCVQKCYCKPQRAQQHSCKMSELSTIT
HOP-1 CASSETTE
COOH

Figure 5.10

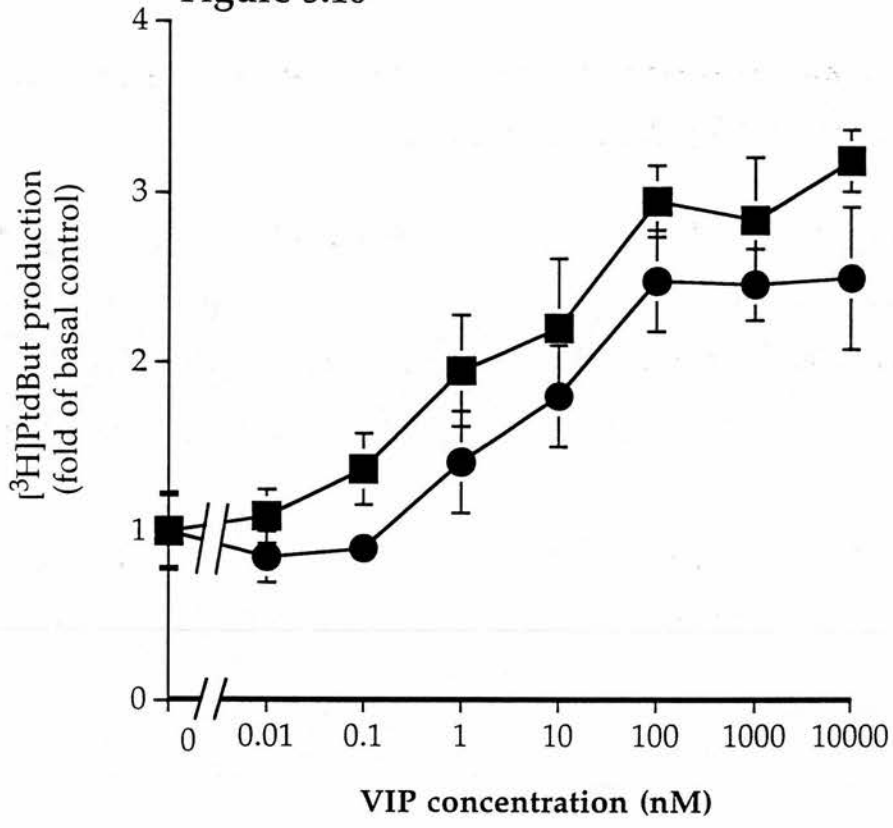
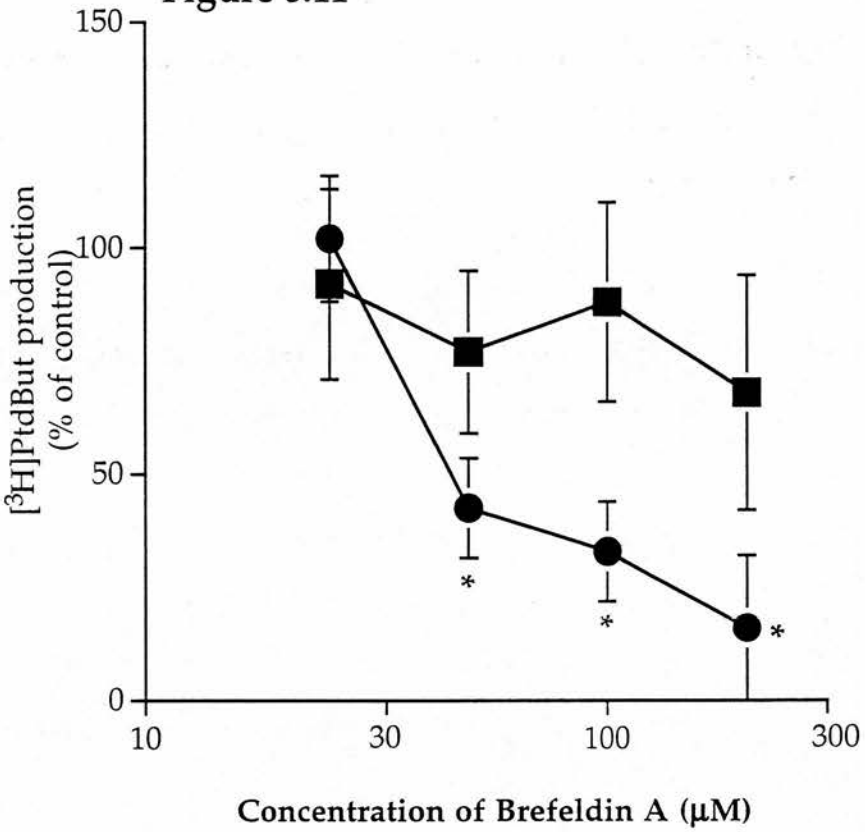

[3H]PtdBut production
(fold of basal control)
4
3
2
1
0
0
0.01
0.1
1
10
100
1000
10000
VIP concentration (nM)

Figure 5.11


150
100
50
0
[3H]PtdBut production
(% of control)
10
30
100
300
*
*
*
Concentration of Brefeldin A (μM)

CHAPTER 6

Overview

Members of the GPCR superfamily play a central role in cellular communications mediating the cell response to numerous hormones, odorants, chemokines, neurotransmitters and light. Through the coupling to heterotrimeric G proteins receptor activation regulates numerous intracellular effector molecules including ion channels, adenylate cyclase and phospholipases A_2, C and D. The conservation of PLD-like enzymes across species, the ubiquitous distribution of the enzyme in mammalian tissues and the activation by a vast array of agonists (Bocckino, S. B. and J. H. Exton 1996, Exton, J. H. 1997, Hammond, S. M., et al. 1995, Ponting, C. P. and I. D. Kerr 1996), points to a critical role in the signalling of a immense array of extracellular stimuli. The stimulation of a receptor with ligand is proposed to cause a conformational change and expose buried regions of the receptor which are involved in G protein coupling, such as the second and third intracellular loops (Altenbach, C., et al. 1996, Bukusoglu, G. and D. D. Jenness 1996, Farahbakhsh, Z. T., et al. 1993, Farahbakhsh, Z. T., et al. 1995, Farrens, D. L., et al. 1996, Gether, U., et al. 1997, Luo, X., et al. 1994). By using mutagenesis of amino acid residues within receptors and the construction of chimaeric receptors numerous investigators have isolated the receptor determinants that control coupling to G proteins, the i2 and i3 loops are seen as the main G protein coupling domain (reviewed extensively in (Bourne, H. 1997, Gudermann, T., et al. 1996, Gudermann, T., et al. 1997, Savarese, T. M. and C. M. Fraser 1992, Strader, C. D., et al. 1995, Strader, C. D., et al. 1994, Wess, J. 1997). Point mutation of the α_{1B}-adrenergic and M_5 muscarinic acetylcholine receptors in the i3 loop caused constitutive coupling to G protein as seen by the stimulation of the phosphoinositide breakdown in the absence of agonist (Burstein, E. S.,

et al. 1996, Kjelsberg, M. A., et al. 1992). Point mutations in TMD 6 also cause constitutive activation of receptors, for example the mutation of a conserved aspartate residue in TMD 6 of the LH receptor causes constitutive activity and is linked to the disease, familial male precocious puberty (Shenker, A., et al. 1993). The interaction of TMDs 5 and 6 is proposed to be at the centre of this constitutive activity, as a point mutation activating chimaeric FSH and LH receptors with TMDs 5 and 6 sequence from LH but not FSH receptors (Kudo, M., et al. 1996). Furthermore a point mutation in TMDs 5 and 6 from the α_{1A} and α_{1B} respectively, could be silenced by complementary point mutations in TMDs 6 and 5, respectively (Hwa, J., et al. 1996). In addition the mutation of a single residue within the TMD 3 of the α_{1B}-adrenergic receptor caused constitutive activation of G_q but not G_i. Thus the coupling characteristics of a GPCR can be selectively modulated by single amino acids, including those not immediately involved in G protein interaction. The mechanism by which GPCRs stimulate PLD is known to involve the small G proteins ARF and Rho. However the way in which the receptor recruits and interacts with these proteins was unclear. Furthermore the activation of PLD by members of a new family of GPCRs, including receptors for VIP/PACAP/secretin was poorly understood.

The present investigations were designed to characterise the association of ARF and Rho with GPCRs, and their roles in the activation of PLD. The influence of a highly conserved amino acid sequence in TMD 7 of class I receptors, implicated in the maintenance of a receptor conformation was investigated. In a parallel study, the activation of PLD by the class II family of receptors and the significance of the i3 loop structure in this coupling was also scrutinised.

In the 1321N1 cell line, native receptors containing the $NPX_{2-3}Y$ motif in TMD 7: M_3 muscarinic acetylcholine, B_2 bradykinin, H_1 histamine, stimulated PLD in an ARF-dependent manner as shown by the sensitivity of those responses to BFA. The thrombin or thromboxane A_2 receptors ($DPX_{2-3}Y$) receptors were shown to be resistant to BFA and couple to PLD via PLC, as the PLD response was inhibited by the PLC inhibitor U73122 (Chapter 3). The involvement of small G proteins in the activation of PLD by the M_3 but not the thrombin receptor was also established by the inhibition of the carbachol-evoked response by the Rho inhibitor C3 exoenzyme, and a negative RhoA construct. A wild type RhoA construct enhanced PLD stimulation by low concentrations of carbachol but not thrombin. The activation of PLD by either the M_3 or thrombin receptors was not affected by pertussis toxin, suggesting no involvement of proteins from the $G_{i/o}$ family. Using the M_3 and thrombin receptors as typical examples of $NPX_{2-3}Y$ and $DPX_{2-3}Y$ receptors respectively, the interaction of the receptors with the small G proteins ARF and Rho was further investigated. The coupling of the M_3 and not the thrombin receptors to ARF as shown in Figure 3.2, was substantiated by the detection of [^{3}H]NMS but not [^{125}I]Ala-pFPhe-Arg-Cha-HArg-Tyr-NH_2 binding sites in co-immunoprecipitates produced using antibodies to ARF1/3. Moreover the presence of [^{3}H]NMS but not [^{125}I]Ala-pFPhe-Arg-Cha-HArg-Tyr-NH_2 binding sites were detected in co-immunoprecipitates produced using polyclonal antibodies to RhoA. The close association of ARF and RhoA with the M_3 receptor was corroborated by the presence of authentic ARF and RhoA proteins in co-immunoprecipitates produced using polyclonal antibodies to the M_3 receptor solubilized from 1321N1 membranes. The presence of M_3 receptors in ARF1/3 and RhoA immunoprecipitates and ARF and RhoA in M_3 immunoprecipitates was dependent on the pre-

exposure of 1321N1 cells to agonist, suggestive of a translocation of the small G proteins to the membrane where they may interact with relevant targets (Cavenagh, M. M., et al. 1996, Houle, M. G., et al. 1995, Malcolm, K. C., et al. 1996, Rümenapp, U., et al. 1995). The critical involvement of ARF and Rho proteins in the receptor coupling to PLD, as suggested by the results presented in Chapter 3, are consistent with previous investigations of M_3 receptor signalling (Rümenapp, U., et al. 1995, Schmidt, M., et al. 1994, Schmidt, M., et al. 1996). Using anterior pituitary tissue the close association of a native $NPX_{2-3}Y$-containing and BFA-resistant receptor with ARF was confirmed by the co-immunoprecipitation of ARF with antibodies directed against the AT_1 receptor. The apparent selectivity of receptor interaction with small G proteins was reinforced by using different GTP analogues and the M_3 receptor. GTP analogue activation of trimeric or small G proteins was seen to alter the M_3 receptor affinity for ligand and the activation of PLD, in a manner suggesting the receptor coupling to PLD via small G proteins.

The GnRH receptor is unusual in that it contains an arrangement of conserved aspartate and asparagine residues in TMDs 7 and 2, that is not found in any other known class I GPCR (Illing, N., et al. 1992, Probst, W. C., et al. 1992, Reinhart, J., et al. 1992, Tsutsumi, M., et al. 1992, Zhou, W. C., et al. 1994). The role of the $N/DPX_{2-3}Y$ motif in GPCR-coupling to PLD was characterised by the use of the wild type ($DPX_{2-3}Y$) and Asn318 mutant GnRH receptor where the aspartate in TMD 7 was replaced with an asparagine residue thereby restoring the conserved $NPX_{2-3}Y$ sequence. In agreement with the results in Chapter 3, the Asn318 mutant but not the wild type GnRH receptor activated PLD in an BFA-sensitive manner.

A wild type and mutant 5-HT_{2A} receptor which had the asparagine residue in TMD 7 replaced with an aspartate, a mutation reciprocal to that carried out on the GnRH receptor, were used to consolidate the importance of the conserved N/DPX_{2-3}Y motif in the receptors coupling to PLD. The wild type but not the mutant 5-HT_{2A} receptor-induced activation of PLD was sensitive to BFA. The wild type GnRH receptor akin to the thrombin and TXA_2 receptors stimulated PLD in an U73122-sensitive manner. Furthermore the mutation of the Asp at position 318 to asparagine appears to strengthen the coupling of the mutant GnRH receptor to PLD, as observed in the increased rates for the activation of PLD at short time points (Chapter 4). The dramatic changes in the activation of PLD seen after the mutation of the conserved aspartate residue in TMD 7 were not paralleled by changes in receptor-induced breakdown of phosphoinositides. Consistently with the co-immunoprecipitation results from Chapter 3, the Asn318 mutant but not the wild type GnRH receptor was found in co-immunoprecipitates produced using the polyclonal ARF1/3 and RhoA antibodies. As with the work on the M_3 receptor, the interaction of ARF and Rho proteins with the GnRH receptors required the pre-exposure to agonist. Therefore the hypothesis that there is a close interaction of GPCRs with ARF and Rho proteins in the activation of PLD was substantiated, and a requirement for NPX_{2-3}Y in TMD 7 was indicated. Various investigators have proposed a network of inter-helical H-bond interactions, and that the Asn or Asp in the N/DPX_{2-3}Y motif and the conserved tyrosine participate in this network (Ballesteros, J., et al. 1998, Fanelli, F., et al. 1995, Hunyady, L., et al. 1995, Konvicka, K., et al. 1998, Liu, J., et al. 1995, Oliveira, L., et al. 1994, Perlman, J. H., et al. 1996, Scheer, A., et al. 1996). The N/DPX_{2-3}Y sequence appears to participate in the control of receptor activation and

subsequent coupling to G proteins as has been suggested by both mutagenesis and computer modelling (Fanelli, F., et al. 1995, Konvicka, K., et al. 1998, Oliveira, L., et al. 1994, Perlman, J. H., et al. 1996, Scheer, A., et al. 1996, Scheer, A., et al. 1997) .

The activation of PLD by receptors from the class II family was first shown by investigations of the glucagon receptor (Pittner, R. A. and J. N. Fain 1991). However this report was a preliminary study that has not been followed up with further investigation of class II GPCR activation of PLD. As shown in Chapter 5 there was a potent and concentration-dependent activation of PLD by VIP_1, VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors expressed in CHO cells. ARF appears to be involved in the stimulation of PLD by the VIP_1, VIP_2 and $PACAP_{long}$ receptors, while the $PACAP_{short}$ receptor couples to PLD via PLC, as shown by U73122 blockade of its stimulation of PLD. The stimulation of cAMP production by VIP_1, VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors was comparable; furthermore the stimulation of phosphoinositide hydrolysis by the $PACAP_{short}$ and $PACAP_{long}$ receptors was also similar. Therefore the differences in i3 loop structure in the $PACAP_{short}$ and $PACAP_{long}$ receptors apparently affect only the stimulation of PLD. The dramatic differences in the activation of PLD by the $PACAP_{short}$ and $PACAP_{long}$ can only be attributed to the effect the presence of the 28 amino acid hop-1 insert in the $PACAP_{long}$ receptor.

By constructing chimaeric receptors carrying the i3 loop of the $PACAP_{short}$ or $PACAP_{long}$ receptors, replacing that of the VIP_2 receptor, we investigated the role of i3 loop in the activation of PLD. The $VIP_2/PACAP_{long}$ but not the $VIP_2/PACAP_{short}$ receptor activates PLD in an ARF-dependent manner. The $PACAP_{short}$ receptor appears to be the only receptor studied here that cannot couple to PLD via ARF, with this

in mind we analysed the sequence of the receptors in an attempt to discover the nature of this difference. The VIP_2 receptor contains a classical BBXXB motif at the i3/TM6 junction which has been identified by the work of Nishimoto's group as a potential activator of G proteins (Ikezu, T., et al. 1992). The VIP_1 and $PACAP_{long}$ form receptors contain basic amino acids in a similar spaced motif to that present in the α_2-adrenergic receptor at a position beginning 14-18 amino acids upstream of TMD 6, as detailed by the work of Burstein *et al.*. (Burstein, E. S., et al. 1995). The $PACAP_{short}$ receptor has no recognised conserved G protein coupling motif in its i3 and this may conceivably be a reason for its lack of coupling to PLD via ARF.

The activation of PLD by GPCRs has long been known to involve ARF and Rho proteins (Bowman, E. P., et al. 1993, Brown, H. A., et al. 1993, Cockcroft, S., et al. 1994). The recent cloning of the human and rodent forms of PLD1 has confirmed this involvement (Hammond, S. M., et al. 1995, Hammond, S. M., et al. 1997, Park, S.-K., et al. 1997). However the mode of interaction of the receptor with the G protein was not clear. It appears from the data presented in this study that the rhodopsin family of GPCRs can form functional complexes with the small G proteins ARF and Rho. There is a close association of receptor with ARF and Rho that has never previously been reported, although the analogous association of GPCRs with trimeric G proteins is well reported (Georgoussi, Z., et al. 1995, Okuma, Y. and T. Reisine 1992, Raymond, J. R., et al. 1993). We found that the formation of this association is dependent on the presence of the highly conserved $NPX_{2-3}Y$ motif in TMD 7. The receptors containing the non-canonical $DPX_{2-3}Y$ sequence appear to select a PLC-dependent pathway for the activation of PLD. The class II family of

proteins can employ the same ARF-dependent mechanism for the activation of PLD seen in their class I cousins, with a similar selectivity at the receptor level for an alternative PLC-dependent pathway if ARF-association is prohibited by receptor features (Chapter 5).

The method of association of the small G proteins with receptor is unclear although obviously close, and may involve the numerous cofactors that have been reported including the ARF guanine exchange factor, and a Rho-associated 50 kDa factor (Chardin, P., et al. 1996, Kwak, J. Y., et al. 1995, Morinaga, N., et al. 1996, Randazzo, P. A. and R. A. Kahn 1994). The precise site of receptor and small G protein coupling is unknown but the results from Chapters 3, 4, and 5 and the literature suggest that the determinants controlling receptor coupling reside in the i3 loop or immediately adjacent areas which control the receptor conformation. Results from this study provide a starting point for the delineation of the domains and processes directly involved in controlling GPCR activation of PLD.

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Publications

Rhodopsin-family receptors associate with small G proteins to activate phospholipase D

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G-protein-coupled receptors of the rhodopsin family transduce many important neural and endocrine signals. These receptors activate heterotrimeric G proteins and in many cases also cause activation of phospholipase D, an enzyme that can be controlled by the small G proteins ARF and RhoA[1–3]. Here we show that the activation of phospholipase D that is induced by many, but not all, Ca^{2+}-mobilizing G-protein-coupled receptors is sensitive to inhibitors of ARF and of RhoA. Receptors of this type were co-immunoprecipitated with ARF or RhoA on exposure to agonists, and the effects of GTP analogues on ligand binding to the receptor changed to a profile that is characteristic of small G proteins. These receptors contain the amino-acid sequence Asn-ProXXTyr in their seventh transmembrane domain, whereas receptors capable of activating phospholipase D without involving ARF contain the sequence AspProXXTyr. Mutation of this latter sequence to AsnProXXTyr in the gonadotropin-releasing hormone receptor conferred sensitivity to an inhibitor of ARF, and the reciprocal mutation in the 5-HT_{2A} receptor for 5-hydroxytryptamine reduced its sensitivity to the inhibitor. Receptors carrying the AsnProXXTyr motif thus seem to form functional complexes with ARF and RhoA.

The activation of phospholipase D (PLD) (measured as formation of [^{3}H]phosphatidylbutanol) by a number of receptors that are native to 1321N1 human astrocytoma cells[4] was differentially inhibited by brefeldin A (BFA), an inhibitor of guanine-nucleotide exchange on ARF[5]. Activation of PLD by H_1 histamine, B_2 bradykinin and M_3 muscarinic receptors in these cells[6] (as for M_3 receptors expressed in HEK 293 cells[7]) was sensitive to BFA whereas activation of PLD by thrombin and thromboxane A_2 receptors was BFA-resistant (Fig. 1a). Thrombin- but not M_3-receptor responses were attenuated by the phospholipase C (PLC) inhibitor U73122, but neither response was affected by pertussis toxin (Fig. 1b). The Rho inhibitor C3 exoenzyme and a negative construct, CMV5 Asn 19 RhoA (ref. 8), both reduced M_3- but not thrombin-receptor-mediated activation of PLD, whereas wild-type RhoA increased responses to a lower concentration of carbachol but not thrombin (Fig. 1b). These results indicate that some Ca^{2+}-mobilizing G-protein-coupled receptors (GPCRs) use a pathway of PLD activation that is independent of Gq/11 (which activates PLC) and Gi/o, yet involves ARF and RhoA.

We used co-immunoprecipitation to test whether M_3-receptor-mediated PLD activation might involve a step in which the receptor and small G proteins interact closely. Figure 1c shows that solubilized M_3 receptors (levels of which were measured by binding of [^{3}H]*N*-methyl scopolamine ([^{3}H]NMS)) could be immunoprecipitated using polyclonal antibodies against ARF1/3 (ref. 9) or RhoA[10]. Yields from using combinations of antibodies were less than additive. Co-immunoprecipitation required pre-exposure to agonists (priming) and there was little co-imunoprecipitation when

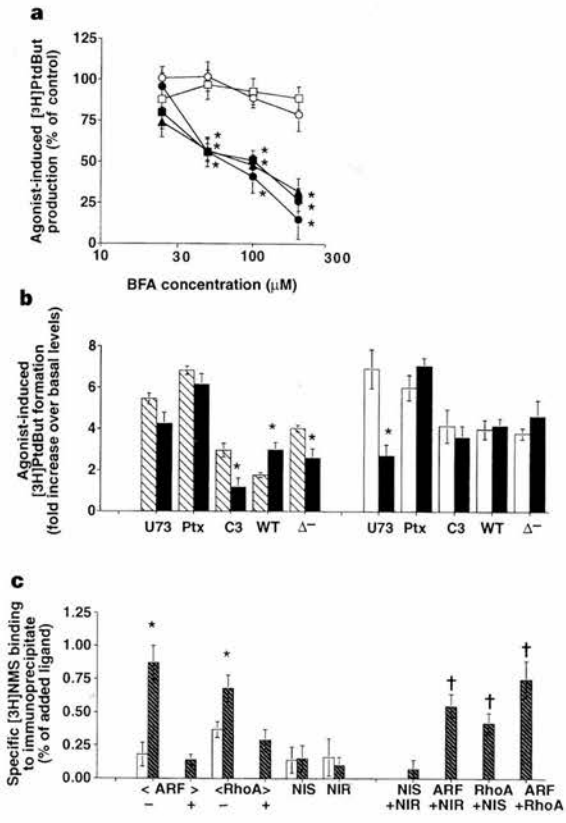

Figure 1 Properties of agonist-evoked PLD responses in 1321N1 cells and co-immunoprecipitation of M_3 receptors with ARF1/3 and RhoA antibodies. **a**, the effects of BFA on [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut) production elicited by: ● 200 μM carbachol, ■ 10 μM bradykinin, ▲ 2 mM histamine, ○ 0.5 units per ml thrombin, or □ 30 μM U46619 (a TXA_2-receptor agonist). IC_{50} values for effects of BFA on M_3, bradykinin and histamine responses were 72 ± 11, 78 ± 15 and 82 ± 13 μM, respectively, whereas thrombin-receptor and TXA_2-receptor responses were resistant up to 200 μM BFA. **b**, Production of PtdBut in response to carbachol and thrombin is shown by hatched and open columns, respectively. Corresponding values for reagent-treated cells are shown by adjacent black columns. Reagents were U73122 (20 μM; U 73), pertussis toxin (200 ng ml^{-1} for 18 h; Ptx), C3 exoenzyme (4.8 μg ml^{-1}; C3) and wild-type or negative RhoA constructs (WT and Δ$^-$). Carbachol was used at a concentration of 200 μM except in C3 and WT RhoA experiments, where 100 μM and 20 μM, respectively, were used. Thrombin was usually 0.5 units per ml but 0.2 units per ml in WT RhoA experiments. In **a**, **b**, values are means ± s.e.m.; $n = 4$–10; significant changes from the control levels are indicated by asterisks ($P < 0.05$; Wilcoxon test). **c**, Solubilized membrane proteins from cells preincubated with carbachol (hatched columns) or from controls (open columns) were immunoprecipitated with sheep anti-ARF1/3 antibody (ARF), rabbit anti-RhoA antibody (RhoA), non-immune sheep IgG (NIS) or non-immune rabbit IgG (NIR) or combinations of these antibodies, before labelling M_3 receptors with [^{3}H]NMS. In some ARF/RhoA immunoprecipitations, blocking peptides were included (shown as ±). Values are means ± s.e.m. ($n = 6$–9). For asterisked bars, $P < 0.05$ compared with unprimed controls, and for bars with daggers $P < 0.05$ compared with non-immune IgG controls (Wilcoxon test). We estimate that up to 48% and 27% of ligand binding to solubilized M_3 receptor may be associated specifically (in a peptide-blockable manner) with ARF and RhoA immunoprecipitates after agonist priming.

using control immunoreagents or in the presence of excess peptide antigen. Use of BFA (100 μM) during priming reduced the yield of M_3 receptors in ARF1/3 immunoprecipitates by 74 ± 19%. Thrombin receptors, labelled with [^{125}I]Ala-pFPhe-Arg-Cha-HArg-Tyr-NH_2 ([^{125}I]TRP; ref. 11) could not be co-immunoprecipitated by antibodies against ARF/Rho after priming with thrombin agonist (data not shown). When a control immunoprecipitating antibody (anti-PKCα, Transduction Laboratories) was used to collect another signalling protein known to translocate to 1321N1 cell membranes, preincubation with carbachol caused no increase in [^{3}H]NMS binding to immunoprecipitates despite a 3.1 ± 0.3-fold increase in membrane binding of [^{3}H]phorbol 12,13-dibutyrate (data not shown).

Immunoblotting using distinct (monoclonal) antibodies showed that authentic ARF and Rho were present in ARF/Rho-directed immunoprecipitates from carbachol-primed cells; ARF and Rho were not present in immunoprecipitates when peptide antigens to the polyclonal reagents were added (Fig. 2a, b). In the detergent/NaCl conditions that we used, the monoclonal anti-ARF antibodies ID9 (ref. 12) and clone 26 (Transduction Laboratories) were much less effective or completely ineffective, respectively, at precipitating ARF immunoreactivity and [^{3}H]NMS-binding sites (data not shown). An M_3-receptor antiserum fraction[6], but not non-immune IgG, caused co-precipitation of immunoreactive ARF and RhoA after priming with carbachol (Fig. 2c, d). Similarly, polyclonal antibodies to the AT_1 receptor (BFA inhibited PLD activation by this receptor; half-maximal inhibitory concentration (IC_{50}) 58 ± 5 μM) caused priming-dependent, peptide-blockable co-precipitation of authentic ARF (Fig. 2e) and Rho (data not shown). In addition, when AT_{II}-primed membranes were treated with the crosslinker ethylene glycolbis(succinimidylsuccinate) and immunoprecipitations carried out with antibody ID9 in the presence of Triton X-100/SDS, specific AT_1-receptor immunoreactivity became detectable in immunoprecipitated high-molecular-mass complexes of approximately 170K (data not shown). These results indicate that certain receptors may be able to form physical complexes involving ARF1/3 and/or RhoA and that this may be important in the activation of PLD.

We used GTP analogues to determine whether the effects of these analogues on both receptor and effector properties were consistent with the above hypothesis. The affinity of M_3 receptors labelled with [^{3}H]NMS for carbachol was reduced by guanosine 5′-0-(3-thio)-triphosphate (GTPγS), BeF_3^- (an isostere of AlF_4^- (ref. 13)) and guanosine 5′-[βγ-methylene]triphosphate ($GPPCH_2P$) (Table 1a).

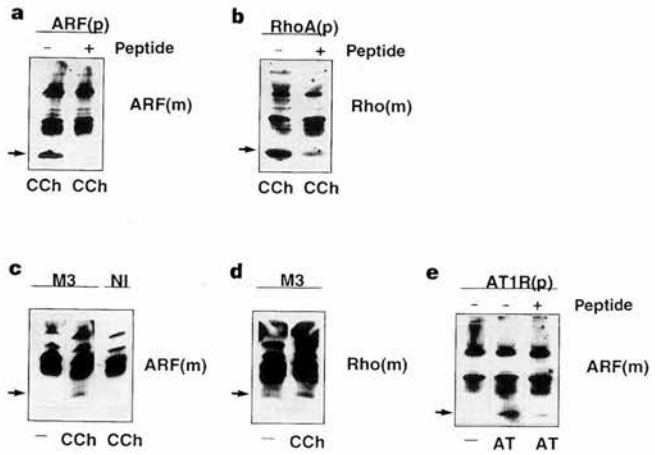

Figure 2 Immunoblots for ARF and Rho on immunoprecipitates generated with polyclonal ARF1/3, RhoA and receptor antibodies. Blots **a**–**d** are from 1321N1 cells and **e** is from rat anterior pituitary cells. For each panel, the labels below indicate the preincubation conditions (CCh, carbachol; AT, ATII; -, control. Labels above show the immunoprecipitation reagents (ARF(p), sheep polyclonal anti-ARF1/3 antibody; RhoA(p), rabbit polyclonal anti-RhoA antibody; M_3, rabbit polyclonal anti-M_3-receptor antibody; NI, non-immune rabbit IgG; AT1R(p), rabbit polyclonal anti-AT_1-receptor antibody), with (+) or without (–) blocking peptides. Labels to the right indicate the immunoblotting reagents (ARF(m), mouse monoclonal anti-ARF antibody; Rho(m), mouse monoclonal anti-Rho antibody). The arrow indicates the position of the 20 kDa molecular mass standard.

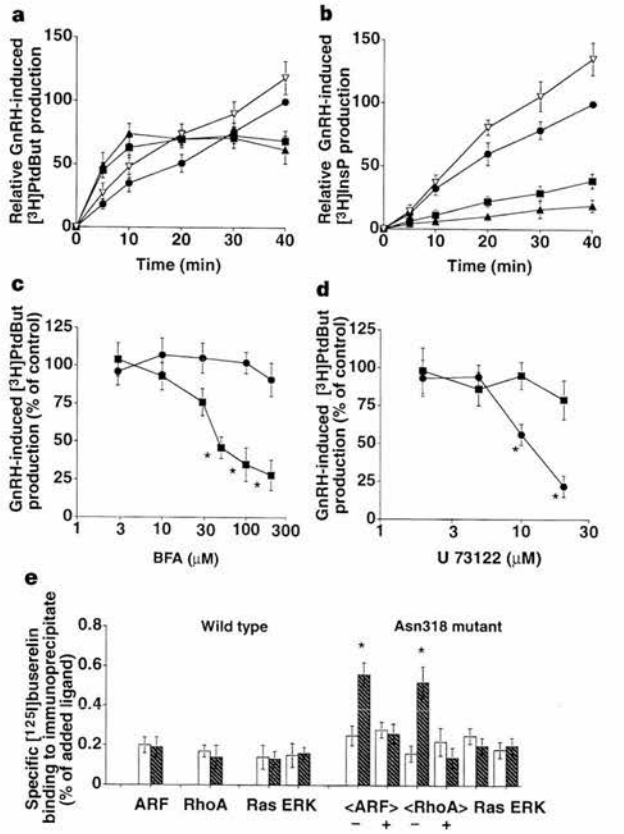

Figure 3 PLD and PLC responses of wild-type, Asn 318 and Asp 318 plus Asp 87 Asn 318 mutant GnRH receptors expressed in COS 7 cells and receptor immunoprecipitation with ARF1/3 and RhoA antibodies. **a**, **b** Show the time course of [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut) and [^{3}H]inositol phosphate ([^{3}H]InsP) production evoked by 100 nM GnRH at ● wild-type, ■ Asn 318 mutant and ▲ Asp 87 Asn 318 mutant receptors and by ∇ AlF_4^- (10 mM NaF and 30 μM $AlCl_3$) in Asn 318 mutant expressing cells. Values are percentages of wild-type response at 40 min (means ± s.e.m.; n = 4–6). **c**, **d** Show effects of BFA and U73122 on [^{3}H]PtdBut production evoked by 100 nM GnRH at ● wild-type and ■ Asn 318 mutant receptors. Values are means ± s.e.m.; n = 4–6. **e**, Shows binding of [^{125}I]buserelin to immunoprecipitates from cells expressing wild-type or Asn 318 mutant receptors. Extracts from cells preincubated with GnRH (hatched columns) or from controls (open columns) were immunoprecipitated with antibodies against ARF1/3 (ARF), RhoA (RhoA), p21Ras (Ras) or ERK1/2 (ERK). Blocking peptides for ARF/RhoA antibodies were sometimes included (indicated as ±). Values are means ± s.e.m.; n = 4–8. Asterisks relate to P values of <0.05 compared to controls, by Wilcoxon test. After agonist priming, 26% and 33% of ligand binding to solubilized mutant GnRH receptor (1100–1400 c.p.m. per sample) seemed to be associated specifically (in a peptide-blockable manner) with ARF and RhoA immunoprecipitates whereas specific association with these precipitates of the wild-type was negligible.

After carbachol priming, the effect of GTPγS was unaltered, that of BeF_3^- was attenuated and that of $GPPCH_2P$ was increased in a way that was 57 ± 10% inhibited by BFA. Table 1b shows that GTP analogues activated PLD in permeabilized cells and that their effects were modified by agonist priming[14–16]. After carbachol priming of 1321N1 cells, the effect of GTPγS did not change significantly but that of BeF_3^- was reduced and that of $GPPCH_2P$ was greatly increased. Agonist priming lowered the effective concentration for half maximum response (EC_{50}) for $GPPCH_2P$ from 367 ± 74 μM to 97 ± 11 μM (6% and 22%, respectively, of the potency of GTPγS (itself with an unaltered EC_{50} of 21–22 μM)). Agonist exposure causes ARF1/3 and RhoA[7,16,17] to translocate to cell membranes and the increased effect of $GPPCH_2P$ here was abrogated by BFA or C3 exoenzyme (Table 1b). Effects of AlF_4^- and BeF_3^- may involve trimeric, but not small, G proteins[13,18] (although selectivity may be modified *in vivo*). In contrast, βγ-methylene analogues of GTP may retain a substantial fraction of the potency of GTPγS at ARF, Rab5 and other small G proteins, despite being very weak activators of trimeric G proteins and Ras[19,20]. Indeed in an assay of [^{35}S]GTPγS association to native ARF, collected from ID9 immunoprecipitates (which were later renatured) of urea-treated extracts from carbachol-primed 1321N1-cell membranes, $GPPCH_2P$ retained 30% of the potency of GTPγS with mean IC_{50} values of 54 and 16 μM, respectively (R.M. and D.M., unpublished observations). These results concur with the idea that agonist-induced translocation of ARF/RhoA to membranes may promote their involvement in some form of complex with GPCRs, and may ultimately enhance receptor-mediated activation of PLD.

Each of the receptors that were sensitive to BFA, C3 exoenzyme or CMV5 Asn 19 RhoA or that could be co-immunoprecipitated with ARF 1/3 or RhoA contains the canonical AsnProXXTyr motif (where X represents any amino acid) in transmembrane domain VII (TMD VII), whereas the other receptors contain AspProXXTyr instead. The wild-type gonadotropin-releasing hormone (GnRH) receptor (which contains AspProXXTyr) and two mutants containing an AsnProXXTyr motif (containing mutation of Asp 318 → Asn or Asn 87 → Asp as well as Asp 318 → Asn (ref. 21)) were expressed in COS 7 cells. The wild-type receptor activated PLD linearly over 30–40 min, like the receptor in αT3-1 cells[22]. In contrast, the Asn 318 and Asp 87 Asn 318 mutants displayed initial rates of [^{3}H]phosphatidylbutanol formation that were more than 2.5-fold that of the wild-type receptor (Fig. 3a). Enhanced PLD coupling in the mutants was observed despite reduced [^{3}H]inositol phosphate production (Fig. 3b) and reduced membrane [^{125}I]buserelin binding (for which values of 589 ± 39, 230 ± 41 and 148 ± 36 fmol per mg protein B_{max} were obtained for cells expressing wild-type, single mutant and double mutant receptors, respectively). PLD-activation responses of the mutants desensitized rapidly, whereas PLC-activation responses did not. In contrast, postreceptor stimulation of trimeric G proteins with AlF_4^- caused non-desensitizing activation of both PLD and PLC in Asn 318 mutant cells. Neither altered receptor kinetics nor enhanced receptor internalization to allow contact with intracellular PLD[23] could explain the enhanced PLD coupling, as [^{125}I]buserelin association with the membrane and receptor internalization rates in wild-type and Asn 318-mutant cells were indistinguishable. In addition, 200 μM monodansylcadaverine and 30 μM monensin (which caused 70–80% inhibition of receptor internalization) increased rather than reduced, both PLD responses (data not shown).

PLD activation by the wild-type receptor was inhibited by U73122 (IC_{50} of 11 ± 1 μM) but not by BFA in concentrations up to 200 μM (these data are similar to data from αT3-1 cells), whereas the Asn 318-mutant response was sensitive to BFA (IC_{50} of 54 ± 8 μM) but not U73122 in concentrations up to 20 μM (Fig. 3c, d). ATP, an agonist for the native P_{2u} receptor (which contains the AspProXXTyr motif), and ionomycin/phorbol ester both caused BFA-resistant PLD activation (data not shown). The gain of BFA-sensitive PLD (but not PLC) activation upon replacement of the AspProXXTyr motif with AsnProXXTyr indicates that this structural motif may be important in gating ARF/Rho-mediated coupling to PLD. This hypothesis is supported by the greater sensitivity to BFA (IC_{50} 47 ± 11 μM) of PLD activation by the wild-type 5-HT_{2A} receptor (which contains AsnProXXTyr) than its Asp 376 mutant (ref. 24) (24 ± 13 inhibition at 200 μM BFA) expressed in COS 7 cells.

The importance of the AsnProXXTyr motif in the proposed linkage between receptor and small G proteins was directly indicated by co-immunoprecipitation of agonist-treated Asn 318 mutant but not wild-type GnRH receptors using ARF and RhoA antibodies (Fig. 3e). Furthermore, although GTPγS increased dissociation of [^{125}I]buserelin from both the Asn 318 mutant and the wild-type receptor in permeabilized cells, $GPPCH_2P$ was effective towards the mutant only and the inverse was true for BeF_3^- (Table 1c). The effect of GTPγS at the mutant, but not the wild-type, receptor was inhibited (by 62 ± 12%) by 50 μM BFA.

Our results suggest a model involving a previously unrecognized association between certain rhodopsin-family receptors and the small G proteins ARF and RhoA. One functional consequence of this appears to be an enhanced coupling of receptors to PLD activation. The interaction seems to occur when an AsnProXXTyr, but not AspProXXTyr, receptor motif is present and may be enhanced by agonist-induced translocation of ARF/RhoA to the plasma membrane. The form and site of interaction of small G proteins with receptors is unknown, but it seems likely that other proteins that act as adapters or regulators of small G protein function[25–28] may participate or mediate in the association. □

Table 1 Effects of GTP analogues on agonist recognition by M_3 and GnRH receptors and on PLD activation

Assay and treatment	GTP analogue		
	GTPγS	F^-	$GPPCH_2P$
(**a**) Carbachol affinity for [^{3}H]NMS-binding sites in 1321N1 cells (fold increase in IC_{50})			
Control	5.23 ± 0.97	4.06 ± 0.80	2.31 ± 0.34
Carbachol-primed	4.48 ± 0.34	1.82 ± 0.31*	3.46 ± 0.40†
(**b**) Activation of PLD in permeabilized 1321N1 cells (Increase over basal activation (%))			
Control	150 ± 9	115 ± 14	48 ± 8
+BFA		117 ± 9	31 ± 3‡
+C3 exoenzyme		95 ± 11	27 ± 5‡
Carbachol-primed	168 ± 10	78 ± 10*	110 ± 12†
+BFA		69 ± 11	38 ± 9‡
+C3 exoenzyme		85 ± 9	23 ± 7‡
(**c**) [^{125}I]buserelin dissociation rate in transfected COS7 cells (Reduction in $t_{1/2}$ of slow component (%))			
Wild-type GnRH receptor	43 ± 4	36 ± 8	12 ± 5
Asn 318 mutant	40 ± 5	7 ± 5*	38 ± 8†

GTPγS, F^- and $GPPCH_2P$ were present at 100 μM, 10 mM and 200 μM, respectively, except in **c** where 3 mM F^- and 100 μM $GPPCH_2P$ were used. BFA and C3 exoenzyme were used at 100 μM and 4.8 μg ml^{-1}, respectively. Values are means ± s.e.m., n = 4–10. Statistically significant differences ($P < 0.05$ by Wilcoxon test): *, less than corresponding unprimed control (**a**, **b**) or wild-type control (**c**); †greater than corresponding unprimed or wild-type control; ‡reversal of GTP-analogue effect.

Methods

Phospholipase assays. PLD and PLC activities were monitored as production of [^{3}H]phosphatidylbutanol and [^{3}H]inositol phosphates, respectively[29]. For assays of PLD activity in acutely permeabilized cells, prelabelled 1321N1 cells were primed with 100 μM carbachol (10 min, 37 °C) or control before intracellular buffer[7] was added. This buffer contained 2 mM MgATP, 10 μM NAD, 6 μM digitonin, 30 mM butan-1-ol, and GTPγS, $GPPCH_2P$ or F^- (NaF in the presence of 30 μM $BeCl_2$) and BFA or C3 exoenzyme as required. Male rat anterior hemipituitaries were labelled for 2 h *in vitro* for assay of AT_{II}-evoked PLD activation.

Liposome treatment and transfection of 1321N1 cells. Cells were treated with lipofectamine containing C3 exoenzyme (2 μg per well, 5 h), CMV5-RhoA constructs (0.5 μg DNA per 4.5 cm^2 well, 7 h) or control[8]. Rho-construct cells were assayed after 48 h.

Solubilization and immunoprecipitation of [^{3}H]NMS-labelled M_3 receptors and [^{125}I]TRP-labelled thrombin receptors. 1321N1 cells (preincubated with carbachol (100 μM) or the thrombin agonist Ser-Phe-Leu-Arg-Asn-NH_2 (30 μM) for 10 min) were homogenized in cold Hepes buffer with peptidase and phosphatase inhibitors. Membranes were solubilized in 5 mM CHAPS, 0.1% Na cholate and 1M NaCl for 30 min at 4 °C. An equal volume of 20% glycerol in CHAPS/cholate buffer without NaCl was added (with 0.6 mg ml^{-1} phosphatidylcholine for M_3 receptors). Supernatant was precleared and incubated (4 °C, 18 h) with sheep anti-ARF1/3 immunoglobulins (10–15 μl/ml; antigen $ARF1_{98-112}$; gift from M. J. O. Wakelam)[9] or with an immunoprecipitating rabbit anti-RhoA IgG (2–3 μg ml^{-1}; antigen $RhoA_{119-132}$; Santa Cruz Biotechnology)[10]. The ARF antiserum immunoprecipitated authentic immunoreactive ARF from CHAPS/cholate/NaCl membrane extracts (Fig. 2a) and from cytosol after its incubation with GTPγS and the addition of CHAPS/cholate/NaCl but not without this treatment (R.M. and M.J., unpublished observations). Blocking peptides were used at 6 μg ml^{-1} and control non-immune IgG at 3 μg ml^{-1}. Immune complexes were collected with protein-G–Sepharose. M_3 receptors were assayed in 10 mM $MgCl_2$, 200 mM NaCl, 10% glycerol, 3 mg ml^{-1} phosphatidylcholine, 20 mM Hepes pH 7.5, 10 nM [^{3}H]NMS (85 Ci $mmol^{-1}$, Du Pont) with or without 10 μM *N*-methyl atropine for 40 min at 37 °C, before precipitation with polyethylene glycol. Thrombin receptors were assayed in 10 mM $MgCl_2$, 150 mM NaCl, 0.25% BSA, 0.05% bacitracin, 0.1 mM 4-(2-aminoethyl)-benzene sulphonyl fluoride (AEBSF), 2 μg ml^{-1} aprotinin, 7% glycerol, 2 mg ml^{-1} phosphatidylcholine, Tris HCl (50 mM pH 7.4), [^{125}I]TRP (ref. 11; 120,000 c.p.m. per assay) with or without 300 nM unlabelled TRP, for 60 min at 4 °C.

Western blotting of immunoprecipitated extracts. Extracts from 1321N1 cells or male rat anterior hemipituitaries (preincubated with 10 μM AT_{II} for 10 min at 37 °C) were immunoprecipitated using polyclonal anti-ARF1/3 antibodies at 20 μl ml^{-1} (peptide at 8 μg ml^{-1}), polyclonal anti-RhoA IgG at 5 μg ml^{-1} (peptide at 20 μg ml^{-1}), rabbit anti-M_3 receptor serum[6] (antigen M_3 $receptor_{561-578}$) (or non-immune rabbit IgG) at 2.5 μg ml^{-1} and polyclonal anti-AT_1 receptor IgG at 5 μg ml^{-1} (antigen AT_1 $receptor_{15-24}$; 20 μg ml^{-1}; Santa Cruz). Proteins collected by protein G beads were separated by SDS–PAGE and blots were incubated with monoclonal anti-ARF IgG (clone 26, 1:200 dilution; Transduction Laboratories), or anti-Rho IgG (clone 26C4, 1:200 dilution; Santa Cruz). Detection was by horseradish-peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

Modulation of agonist affinity at M_3 receptors by GTP analogues. 1321N1 cells were incubated with or without carbachol (20 μM) and/or BFA (100 μM) for 10 min at 37 °C. Carbachol displacement (10–3,000 μM) of membrane [^{3}H]NMS binding was measured in 10 mM $MgCl_2$, 100 mM NaCl, 20 mM Hepes pH 7.5, 1 nM [^{3}H]NMS, with or without GTPγS, $GPPCH_2P$ or F^-, for 60 min at 37 °C.

[^{125}I]buserelin-binding studies in transfected COS 7 cells. Receptor constructs in pcDNA1 (refs 21, 24) were transfected using DEAE dextran (20 μg DNA per 4×10^6 cells) and were assayed 72 h later. Membrane binding was assayed as described[30]. For cell-surface binding, cells in 12-well plates were incubated with ligand, with or without 3 μM GnRH, at 4 °C or 37 °C as appropriate[30]. Internalization was measured at 37 °C for 0–40 min after pre-equilibration with ligand for 90 min at 4 °C. Surface-bound ligand was dissociated with cold 0.2M acetic acid in 0.5M NaCl. Ligand dissociation in permeabilized cells was measured by prelabelling at 4 °C, and then successive incubations with medium containing 22 μM digitonin and GTP analogues at 37 °C. Initial dissociation occurred identically from wild-type and Asn 318 mutant GnRH receptors, reflecting a temperature-dependent reduction in GnRH binding. From 15–50 min, slower dissociation rates were reached and plots of $\ln\frac{B}{B_0}$ against time revealed single linear components of half-lives: wild-type: 22 ± 3 min, and Asn 318 mutant: 26 ± 3 min in controls.

Solubilization and immunoprecipitation of wild-type and mutant GnRH receptors labelled by [^{125}I]buserelin. After preincubating cells with or without 100 nM GnRH for 15 min, membranes were solubilized in 5 mM CHAPS and 1.5M NaCl (ref. 30). Extracts were adjusted to 0.5M NaCl and precleared before incubating (at 4 °C for 18 h) with the polyclonal antibodies anti-ARF1/3 (10 μl ml^{-1}) and anti-RhoA (1 μg ml^{-1}), described above, or with mouse monoclonal anti-Ras IgG (antigen $p21^{ras}$; Transduction Laboratories) or mouse monoclonal anti ERK1/2 IgG (antigen $ERK1_{325-345}$; Zymed Laboratories) as controls at 1 μg ml^{-1} (both of which are reported to recognize native conformations of their targets). Blocking peptides for the polyclonal reagents were used at 2 μg ml^{-1}. Immune complexes were collected using protein G–Sepharose, and [^{125}I]buserelin binding was measured in polyethylene glycol precipitates[30].

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ACTIVATION OF PHOSPHOLIPASE D BY THE VIP_2 RECEPTOR AND EVIDENCE FOR ITS ATTENUATION BY PROTEIN KINASE A.

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The recently-cloned type 2 vasoactive intestinal peptide (VIP_2) receptor is a member of the secretin/glucagon/PACAP family of large peptide hormone receptors, characterised by seven transmembrane spanning domains and coupling to heterotrimeric G proteins, but distinct from the rhodopsin family. Members of this family are known to couple to the heterotrimeric G protein, G_s. We have found for the first time that VIP stimulation of the VIP_2 receptor, transiently expressed in COS 7 cells, activates phospholipase D (PLD) which hydrolyses the membrane phospholipid phosphatidyl choline to produce phosphatidic acid and choline.

We have evidence that the PLD response is endogenously attenuated by a protein kinase A-dependent mechanism since PLD activation is greatly enhanced in the presence of the selective protein kinase A inhibitor H89. Similar results were obtained in stably transfected CHO cells where PLD responses were minimal unless protein kinase A was inhibited. Phorbol 12,13-dibutyrate-stimulated PLD activity in CHO cells was also inhibited by the adenylate cyclase activator forskolin.

These results demonstrate that VIP family receptors, specifically the VIP_2 receptor, can activate PLD and that PKA is involved in attenuating or desensitising this response.